Backgrounder on RNA Interference (RNAi)

March 23, 2016

Draft Prepared by Health Canada-Pest Management Regulatory Agency's
Health Evaluation Directorate

Table of Contents

[TOC \O "1-3" \H \Z \U]

Chapter 1: Introduction

Gene silencing is the process of preventing the expression of a certain gene. Ribonucleic acid interference (RNAi) is a post-transcriptional gene silencing process, which is initiated by double-stranded RNA (dsRNA) molecules that inhibit specific gene expression by messenger RNA (mRNA) inactivation (Zamore *et al.,* 2000). The term RNAi was coined by Fire *et al.* in 1998 to describe the observation that dsRNA can block gene expression when it was introduced into *Caenorhabditis elegans* (*C. elegans*).

RNAi has been demonstrated as an important, endogenous pathway used in many different organisms to regulate gene expression post-transcriptionally. Not only is RNAi a vital part of plants' immune response to viruses and bacteria (Stram and Kuzntzova, 2006; Katiyar-Agarwal *et al.,* 2006), it can also produce antiviral responses in other organisms. For example, in both juvenile and adult *Drosophila*, RNAi is important in antiviral immunity and is active against pathogens such as Drosophila X virus (Zambon *et al.*, 2006; Wang X *et al.*, 2006).

Currently, three RNAi mechanisms are known: small-interfering RNA (siRNA), microRNA (miRNA), and Piwi-interacting RNA (piRNA) (Meister, 2013). siRNAs and miRNAs have been better studied, and they base pair with RNA molecules such as mRNAs in a sequence specific fashion in the cytoplasm to interfere with protein synthesis (Meister, 2013). This interference decreases specific protein production and can ultimately induce mortality in target organisms (Nature: RNA interference).

In recent years, RNAi technology has been applied to therapeutic products, such as pharmaceuticals, and more recently to agricultural products. The potential utility of RNAi for insect pest control was first suggested by two studies published in 2006 demonstrating that RNAi can be elicited in insects by oral administration of dsRNA (Araujo *et al.*, 2006; Turner *et al.*, 2006). Subsequently, investigations in the mosquito *Aedes aegypti* provided the first demonstration that RNAi could be induced in insects by topical application of dsRNA (Pridgeon *et al.*, 2008). Currently, there are two main pest control use patterns proposed for RNAi technology:

1) plant-incorporated protectants (PIPs); and
2) non-PIPs, also known as exogenously applied dsRNA products.

1.1 Plant-incorporated protectants (PIPs)

PIPs are genetically modified (GM) plants that produce a continuous supply of pesticidal substances; for RNAi-based PIPs, the protectant is dsRNA. Upon consuming the plant tissues, the dsRNA produced from the plants enter into the insect midgut where it interferes with the production of a vital protein, thereby leading to the target organism's death. Currently, plants may be genetically altered by either nuclear transformation or by chloroplast transformation.

Nuclear Transformation

The most common way to genetically modify a crop plant is by modifying their nuclear genomes to produce dsRNA against specific target gene(s). Baum and colleagues (2007) developed GM corn plants that resisted the western corn rootworm (WCR; *Diabrotica virgifera*). By reducing translation of vacuolar H^+-ATPase subunit A in the pest, the plant increased pest mortality and larval stunting and experienced less root damage as a result. Mao *et al.* (2011) transformed cotton plants to produce dsRNAs that reduced the expression of the P450 gene *CYP6AE14* in cotton bollworms. The reduced P450 activity increased the level of gossypol, an anti-herbivore phytochemical, leading to reduced growth of the larvae. These examples illustrate that the creation of RNAi-based GM crops that are lethal to pests or that deleteriously affect interactions of the pests, however, full protection from herbivory has not been observed. The plant's own RNAi system prevents the accumulation of sufficient amounts of dsRNA to yield such result.

Chloroplast Transformation

Recently, Zhang J *et al.* (2015) performed modifications to the chloroplast genome of plants to allow production of dsRNAs in the chloroplasts. Such plants were dubbed as "transplastomic plants". It was hypothesized that due to chloroplasts' origin[1], a large amount of dsRNA could be produced in the plant. The study showed that transplastomic leaves producing dsRNA caused a mortality rate of 100% in Colorado potato beetle after five days of feeding, a much better result compared to modified nuclear genome.

When comparing chloroplast and nuclear transformation, three major differences are noted:

1) With chloroplast transformation, higher accumulation of intact dsRNA is achievable since chloroplasts do not process dsRNA into siRNAs. In Zhang J's experiment (2015), the beetles that were feeding on the chloroplast-transformed plants ingested almost entirely long dsRNA, whereas beetles feeding on nuclear transformed plants consumed mostly siRNAs. The author explained that long dsRNAs were readily absorbed by the beetle's gut cells, and a strong RNAi response was elicited. The siRNAs either may not be readily absorbed in the gut or are not in a form suitable to induce RNAi effectively.
2) With chloroplast transformation, the potential for transgene spreading is reduced since in most plant species there is no transmission by pollen as most plants chloroplasts are transmitted via maternal plants.
3) However, with chloroplast transformation, the transformation process is very difficult and has not been achieved in most species, unlike nuclear transformation technologies, where there is a much wider range of plants. Developing a protocol often requires significant efforts to optimize tissue culture, regeneration and selection procedures (Bock, 2014). Workable transformation protocols for important model plants (including *Arabidopsis thaliana*) and agriculturally important staple crops (including all cereals) are still lacking and sometimes even switching to a closely related species can be challenging (Bock, 2014).

[1] free-living cyanobacteria that lack an RNAi system

Many GM food and feed crops using RNAi-based technology have been approved throughout the years globally (Appendix I), as the risks of RNAi were previously considered to be minimal because no new proteins were produced (Then, 2015). However, a recent controversial study by Zhang L *et al.* (2012) suggested that dietary plant miRNAs could pass through mice gastrointestinal (GI) tract and enter the sera and tissues to directly silence an endogenous LDLRAP1 gene in liver and influence cholesterol regulation. The issue of non-target risks was thus raised for RNAi technology.

1.2 Exogenously Applied dsRNA products

Exogenously applied dsRNA products are designed to be applied topically to the surface of crops. The U.S. Environmental Protection Agency (EPA; White Paper, 2013) anticipates that exogenously applied dsRNA products could be applied using the same methods as traditional chemical pesticide. Four likely categories of dsRNA active ingredients present in exogenously applied dsRNA end-use products (EPs) were identified: direct control agents; resistance factor repressors; developmental disruptors; and growth enhancers.

Direct Control Agents

A dsRNA direct control agent is a dsRNA active ingredient that has direct toxic effects upon the pest, resulting in mortality. The family of dsRNA direct control agents likely would include, but is not limited to herbicides, insecticides, and fungicides. This type of dsRNA active ingredient does not depend upon chemical pesticide control, and could be rotated into integrated pest management (IPM) systems to reduce chemical pesticide use and lessen the possibility of resistance development by the target pests.

Resistance Factor Suppressors

A dsRNA resistance factor suppressor is a dsRNA active ingredient that suppresses genetic resistance to a traditional chemical control. This approach is non-toxic as the intent is not to kill the target pest, but to make it vulnerable. This dsRNA active ingredient category does not reduce dependence upon chemical pesticide control, but does permit the continued use of existing chemistry by rendering formerly resistant pests susceptible.

Developmental Disruptors (Growth Regulators)

A dsRNA developmental disruptor is a dsRNA active ingredient that interferes with the normal development or growth of the target pest such that the target pest or its progeny die, are less competitive, or are sterile. Developmental disruptors that are currently registered by the US Food and Drug Administration (FDA) are called insect growth regulators (IGRs), and they fall into two main categories: (1) juvenile hormone mimics (juvenoids) that disrupt hormonal control of larval development and inhibit metamorphosis (e.g., methoprene); and (2) chitin synthesis inhibitors (e.g., triflumuron) that prevent chitin formation and replacement of the old cuticle following ecdysis (molting).

Growth Enhancers

A dsRNA growth enhancer is a dsRNA active ingredient that stimulates, inhibits, or mimics the activity of a naturally-occurring plant hormone. Induced resistance promoters are substances that stimulate the internal defense mechanisms of plants such that they will have an enhanced capacity to resist infection by plant pathogens. It is conceivable that dsRNA active ingredients could be developed to specifically target genes responsible for pathogen resistance. This type of dsRNA product could be used in two ways: (1) stimulate pathogen resistance in desirable food and ornamental plants; and (2) suppress pathogen resistance in weed species. In addition, it is conceivable that a family of dsRNA products could

be developed for the purpose of suppressing disease resistance in other pest taxa (e.g., protection of bees from virus infections).

This backgrounder document will focus on exogenously applied dsRNA products.

Chapter 2: RNA Interference – Overview of relevant pathways

2.1 Gene Silencing Pathways

Currently, three gene-silencing pathways fall under the category of RNAi: siRNA, miRNA and piRNA. Of these three pathways, emphasis will be given to siRNAs as they are often exogenous dsRNAs, while miRNAs are often endogenous and piRNAs are not well characterized.

Small-interfering RNAs (siRNAs)

Insects

In insects, the siRNA precursor is an exogenous dsRNA that is recognized by a protein called Dicer 2 (Dcr-2) and a dsRNA binding protein called Loquaciousin (Loqs) (Figure 1). Dcr-2 has catalytic activity and will cleave the precursor into siRNAs. After cleavage of the dsRNA into siRNAs, the R2D2[2] protein, along with Dcr-2, deliver the siRNAs to the RNA-induced silencing complex (RISC) where the siRNA is bound to the Argonaute 2 (AGO2) protein. The AGO2 protein selects the siRNA strand that is least thermodynamically stable at the 5' end. This strand is the "guide" strand, while the other strand, called the "passenger strand", is degraded by nucleases (Vodovar and Saleh, 2012). The guide strand-AGO2 complex then attaches to the target mRNA with a perfect complementary match, leading to an AGO2-mediated cleavage of the target occurring between the 10th and 11th nucleotide from the 5' end of the guide siRNA. The cleaved target mRNA is then degraded by nucleases (Vodovar and Saleh, 2012), such that no target protein is synthesized which in turn would lead to effects, such as mortality, in the target organisms. In some organisms such as the nematode, *C. elegans*, the cleaved mRNA can serve as a template for RNA-dependent RNA polymerase (RdRP) and cause RNA amplification (see Chapter 5 "*Interspecies variations in RNAi machinery*" more information).

[2] R2D2's name derives from the fact that it contains two dsRNA-binding domains (R2) and is associated with DCR-2 (D2), source: [HYPERLINK "http://www.sdbonline.org/sites/fly/sturtevant/r2d2-1.htm"]

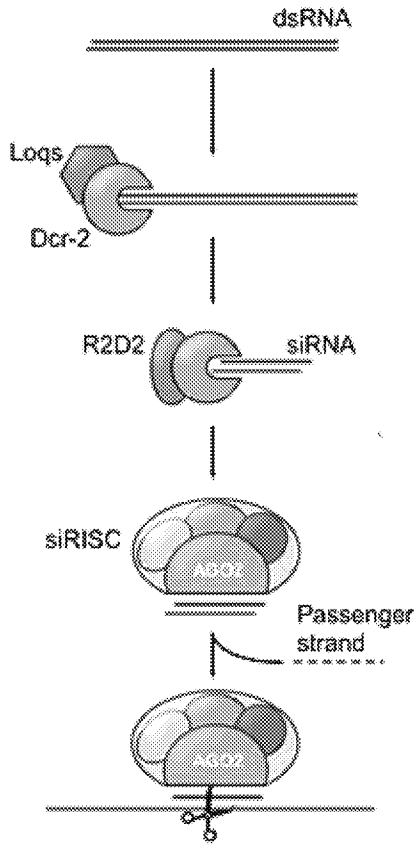

Figure 1. The siRNA silencing pathway in *Drosophila* (Vodovar and Saleh, 2012). The siRNA pathway is initiated by dsRNA which is recognised and cleaved by Dicer 2 (Dcr-2) with the help of Loquaciousin (Loqs). The resulting double-stranded siRNAs are delivered to Argonaute 2 (AGO2)-containing RNA-induced silencing complex (RISC) by Dcr-2 and R2D2. The passenger strand is eliminated and the guide strand directs the degradation of the target RNA via AGO2 catalytic activity.

Mammals

In mammals, a similar mechanism is present; however, the proteins involved in the insect and mammal siRNA pathways are different. The general steps of precursor cleavage, RISC incorporation and cleavage of mRNA remain the same (Figure 2).

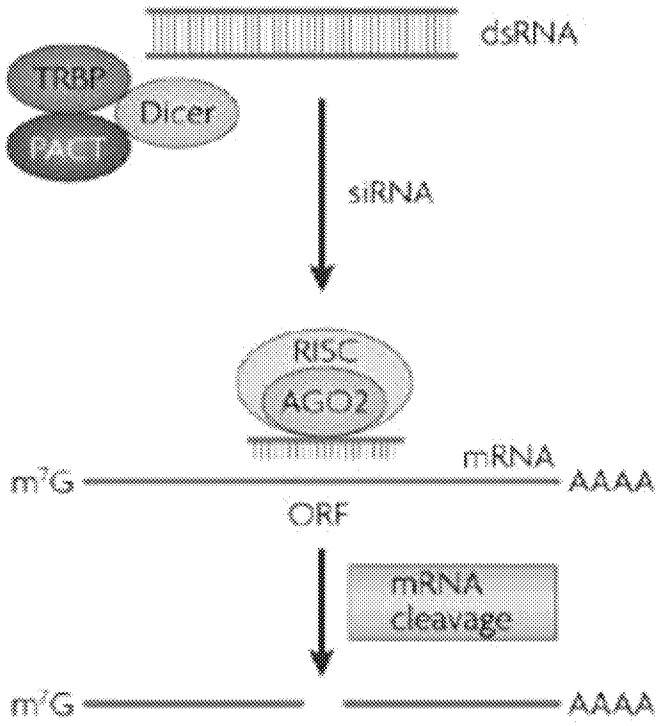

Figure 2. The siRNA silencing pathway in mammalian cells (Kim and Rossi, 2007). Dicer acts together in complex with TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) to cleave the precursor into siRNAs approximately 21 nucleotides long. These shortened RNAs are then incorporated into the RNA-induced silencing complex (RISC). At the heart of RISC, the siRNA binds to a highly conserved Argonaute protein (AGO). Guide strand is selected based on thermodynamics. The guided strand directs the degradation of the target RNA via AGO mediated cleavage.

Plants

In plants, a similar mechanism as mentioned above is followed. There are three classes of endogenous siRNAs in plants: trans-acting siRNAs (TAS), heterochromatin siRNAs (hcsiRNAs) and natural antisense siRNAs (natsiRNAs) (Nazim Uddin and Kim, 2013). Cleavage of long dsRNAs is mediated by four Dicer-like (DCL) endonucleases (Saumet and Lecellier, 2006). The DCL2 and DCL4 enzymes are believed to be involved in the cleavage of exogenous RNA and in the endogenous TAS pathway (Figure 3).

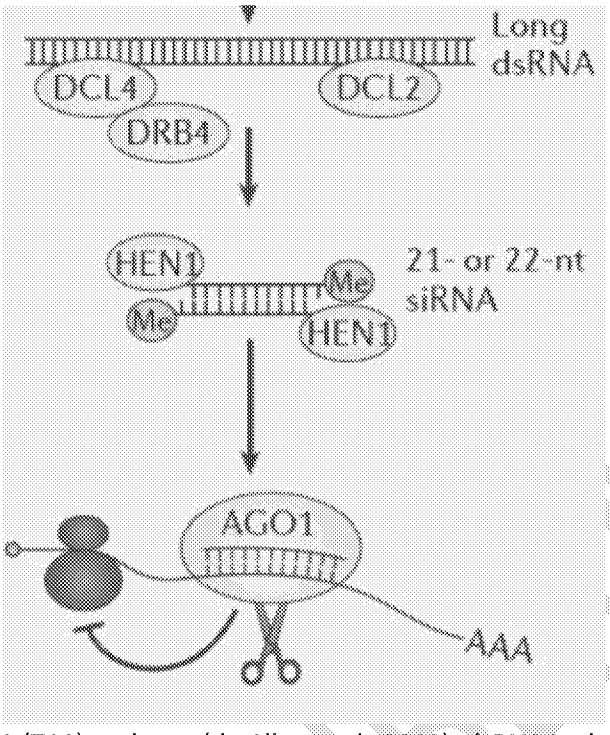

Figure 3. Trans-acting siRNA (TAS) pathway (de Alba *et al.,* 2013). dsRNA is cleaved by Dicer-like proteins (DCL2 and DCL4) into siRNAs with the help of double-stranded-RNA-binding protein 4 (DRB4). Plant siRNAs then undergo 2'-O-methylation by HUA enhancer 1 (HEN1) to prevent degradation. The incorporation of siRNAs into Argonaute of the RISC complex follows the same mechanism as insect and mammalian siRNAs, directing the complex to the target mRNA. In plants, Argonaute protein 1 (AGO1) is responsible for cleavage of the messenger strand or translation repression (Pumplin and Voinnet, 2013).

In plants, RdRPs (including RDR6) can use the cleaved mRNA fragments as templates to synthesize secondary siRNAs (see Chapter 5 "*Interspecies variations in RNAi machinery*" more information).

For the comparison table of the siRNA interference pathways components between insects, mammals, and plants, consult Table 1 of Appendix II.

microRNAs (miRNAs)

Unlike siRNAs, miRNAs are responsible for endogenous gene regulation and are synthesized from their respective genes in the organism's genome as opposed to being generated from exogenous dsRNA or transposable elements (Shabalina and Koonin, 2008). The miRNA biogenesis pathways in plants and animals are distinct (Figure 4.). In animals, miRNAs are pre-processed in the nucleus, exported, and the final 20–23 nucleotides miRNA is produced in the cytoplasm whereas in plants, miRNAs are processed entirely in the nucleus then exported to the cytoplasm. Similar to siRNAs, miRNAs also utilize the RISC complex to target mRNA; however, once incorporated into RISC, the miRNA guide strand does not require perfect base-pairing. Lewis *et al.* (2003) found that exact complementarity between seven of the first eight nucleotides of a miRNA and its target is required for silencing. However, more recent studies found that even though binding of most miRNAs includes the 5′ seed region (nucleotides 2–8 of the guide strand), around 60% of seed interactions contain bulged or mismatched nucleotides (Helwak *et*

al., 2013). This may be explained by the fact that factors such as site context and sequence context contribute to the efficacy of target silencing. For example, Doench and Sharp (2004) observed that the level of expression of both the mRNA and the miRNA, and the binding sites on other mRNAs determined whether the mRNA is regulated or not. Grimson *et al.* (2007) further uncovered five general features of site context in miRNAs that boost site efficacy:

1) AU-rich nucleotide composition;
2) Proximity to coexpressed miRNAs (which leads to cooperative action);
3) Proximity to residues pairing to miRNA nucleotides 13–16;
4) Positioning within the 30 untranslated region (UTR) at least 15 nucleotides from the stop codon; and
5) Positioning away from the center of long UTRs.

Nevertheless, it is generally accepted that the ability of a miRNA to translationally repress a target mRNA is largely dictated by the binding of the first eight nucleotides in the 5′ region of the miRNA.

After binding of the guide strand to the target mRNA, the target mRNA is not necessarily degraded. Target mRNAs can be suppressed by spatially blocking translational unit access to the mRNA, or stored in p-bodies where it can be released in time of stress.

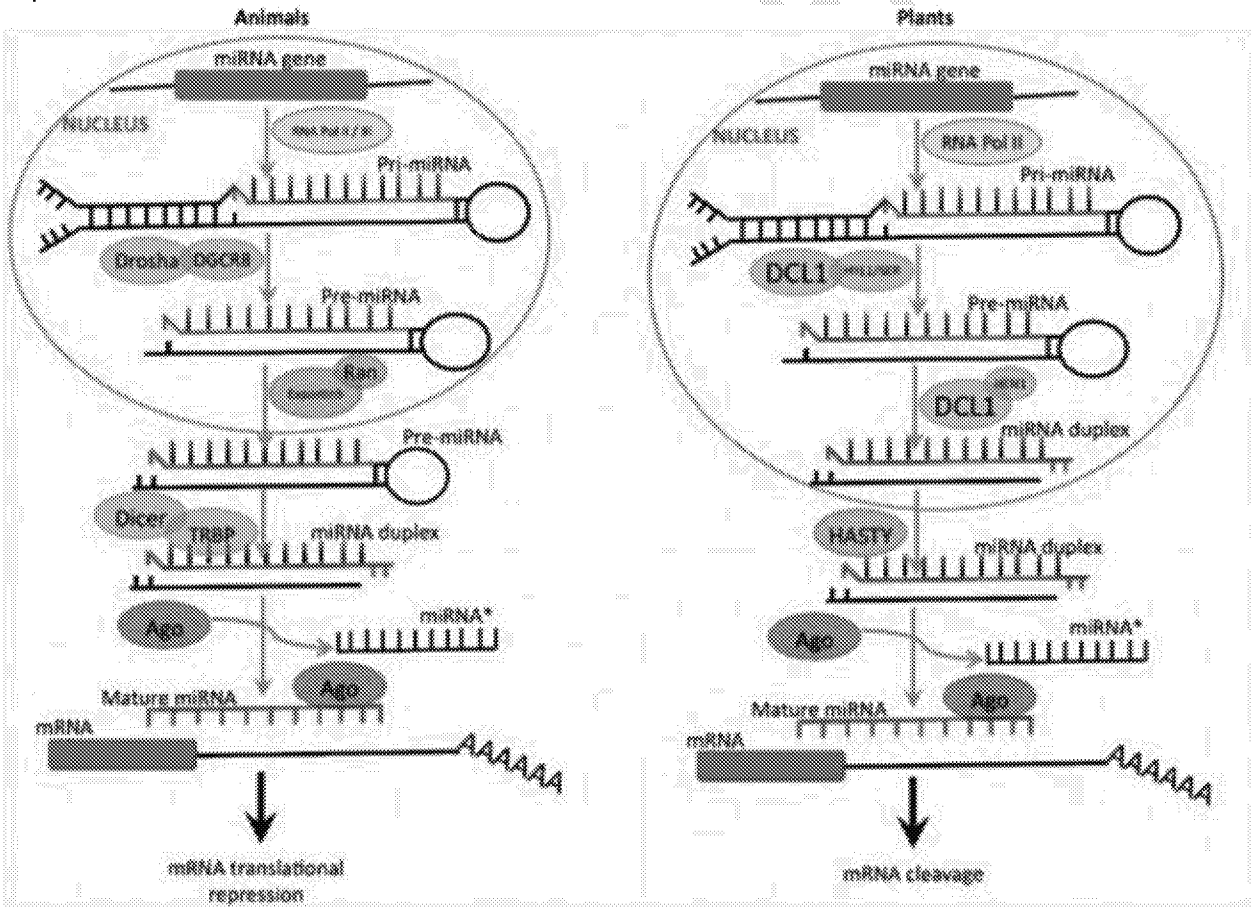

Figure 4. The miRNA pathway in animals and plants (Mallick and Ghosh, 2012). In animals, the miRNA pathway is initiated by the transcription of miRNA genes. Primary miRNA (primiRNA) transcripts are first processed in the nucleus by Drosha and its regulatory subunit DGCR8 then exported to the cytoplasm as pre-miRNAs. There, Dicer and its accessory proteins complete the processing and deliver the mature miRNA to Ago-containing RISC. In plants, primiRNA are excised in the nucleus by Dicer-like 1 (DCL1) with

the help of Hyponastic leaves 1 (HYL1) and Serrate (SE) to generate the miRNA-miRNA* duplex. The miRNA duplex is then exported into the cytoplasm after methylation by methyltransferase HUA enhancer 1 (HEN1). In the cytoplasm, the guide strand associates with Ago1 to form RISC. In both animals and plants, the passenger miRNA* is eliminated and the guide miRNA directs translational repression or cleavage of the target mRNA.

For a comparison table of the miRNA interference pathway components between insects, mammals, and plants, consult Table 2 of Appendix II.

Piwi-interacting RNAs (piRNAs)

Piwi-interacting RNAs (piRNAs) are not as well characterized as miRNAs and siRNAs. These small RNAs in animals are 24 to 32 nucleotides long and interact with the PIWI subfamily of Argonaute proteins. Functions attributed to these molecules include epigenetic regulation, transposon silencing, genome rearrangement and developmental regulation (Ross *et al.,* 2014). The pathways by which piRNAs cause gene silencing are not well understood and will not be the focus of this paper.

2.2 Non-cell-autonomous RNAi: Systemic and Environmental RNAi

In susceptible organisms, RNAi silencing can proceed via cell-autonomous or non-cell-autonomous RNAi. In the case of cell-autonomous RNAi, the silencing process is limited to the cell in which the dsRNA is introduced and encompasses the RNAi process within individual cells. In the case of non-cell-autonomous RNAi, the interfering effect takes place in tissues/cells different from the location of application or production of the dsRNA. There are two different types of non-cell-autonomous RNAi: environmental RNAi and systemic RNAi (Whangbo and Hunter, 2008; Hunter, 2006). Environmental RNAi describes the ability of certain organisms to take up dsRNA from their environment in order to trigger RNA silencing. Systemic RNAi occurs when the silencing phenomenon is locally initiated but spreads from cell to cell throughout the whole organism (Whangbo and Hunter, 2008).

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Environmental RNAi was first observed in *C. elegans*, where RNAi was induced when the nematodes were soaked in a dsRNA solution or fed with bacteria expressing the dsRNA molecules (Meng *et al*., 2013). After soaking wild-type nematodes for 24 hours in a solution of dsRNAs targeting the essential maternal gene *pos-1*[3], Tabara *et al*. (1998) observed that 86% of the F_1 progeny[4] exhibited the distinctive *pos-1* embryonic lethal phenotype. When Fire *et al.* (1998) fed bacteria expressing a dsRNA from the gene *unc-22* to wild-type *C. elegans*, 85% of the nematodes exhibited a partial loss of function for the *unc-22* gene. Studies in *C. elegans* have provided insight into how dsRNA molecules enter an organism from the environment to trigger RNAi. Environmental uptake in *C. elegans* is thought to be

[3] pos-1 is essential for proper fate specification of germ cells, intestine, pharynx, and hypodermis, for more information, visit: [HYPERLINK "http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=worm&q=pos-1"]

[4] *C. elegans* that soaked up the dsRNA targeting pos-1 are themselves unaffected but produce dead embryos with the distinctive pos-1 embryonic lethal phenotype (Tabara et al., 1998).

done via the intestinal lumen while feeding, as *C. elegans* has an impermeable cuticle covering nearly its entire surface (Whangbo and Hunter, 2008). Environmental RNAi in *C. elegans* requires the following steps:

1. dsRNA uptake by the intestinal cells;
2. export of dsRNA or dsRNA-derived silencing signals from the intestinal cells;
3. import of the silencing signals into other tissues (e.g., muscle, epidermis, germline); and
4. targeted gene silencing via the cell autonomous RNAi machinery.

Systemic RNA Interference Deficient 2 (SID-2) has been associated with dsRNA uptake in intestinal cells (Hunter *et al.,* 2006). Other proteins such as SID-3 and SID-5 have also been identified and believed to be involved in endocytosis, however, their exact roles remain unknown (Meng *et al.*, 2013). Environmental RNAi has also been observed in planaria (flatworms), hydra, ticks, honey bees and parasitic nematodes but not in vertebrates (Whangbo and Hunter, 2008). In the honey bee study conducted by Patel *et al.* (2007), a reduction of *Apis mellifera* target of rapamycin (amTOR[5]) by RNAi suppression via the diet was observed in all 10 bees. The resulting[6] bees developed worker morphology instead of queen morphology.

Systemic RNAi was first observed in *C. elegans* when ingested or injected dsRNAs spread throughout the organism and transmitted to its progeny. SID-1 protein acts as a dsRNA channel allowing dsRNA in and out of cells (Hunter *et al.,* 2006). In plants, movement of siRNAs can be either localized via plasmodesmata channels or systemic via the phloem network. Systemic RNAi spread is possible utilizing the RNA-dependent RNA polymerase 6 (RDR6; see Chapter 5 "*Interspecies variations in RNAi machinery*" more information) (Nazim Uddin and Kim, 2013).

[5] amTOR is a nutrient- and energy-sensing kinase that controls organismal growth. High level of amTOR is related to queen bees and low level is correlated to worker bees (Patel *et al.,* 2007).

[6] Reduced growth of the developing larvae (ANOVA: $F_{1,28} = 99.29$, $P<0.00001$), prolonged pre-adult development (ANOVA: $F_{1,19} = 48.00$, $P<0.00001$), reduced wet-weight (size) at adult emergence (ANOVA: $F_{1,19} = 68.28$, $P<0.00001$)

Chapter 3: dsRNA Mass Production

Single-stranded RNAs (ssRNAs) hybridization

In theory, dsRNA can be synthesized using genetically engineered microorganisms, chemical synthesis or extraction methods; however yields are likely to vary. Current mass production methods utilize bacteria for synthesis of dsRNAs. Ongvarrasopone *et al.* (2007) produced dsRNA in bacteria by first cloning complementary DNA (cDNA) of the desired gene in both orientations into a suitable plasmid under a T7 promoter (Figure 5) and then inserting the plasmid into the bacteria[7]. RNA production was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) using bacterial T7 RNA polymerase where sense and antisense ssRNAs were synthesized and annealed to yield dsRNA (Ongvarrasopone *et al.,* 2007).

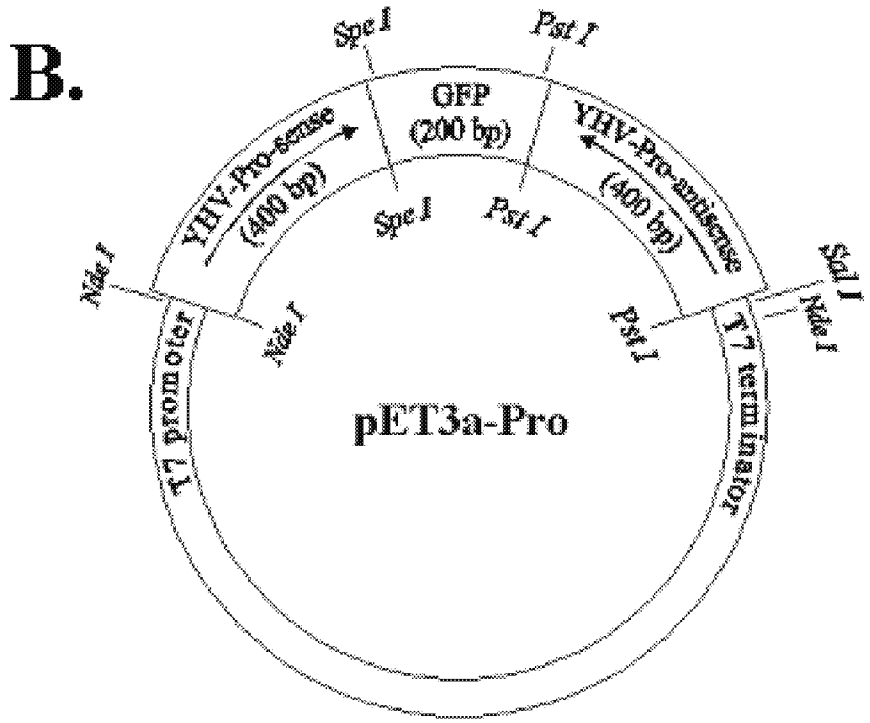


Figure 5. Diagram of pET-3a-Pro plasmid DNA construct for *in vivo* expression in HT115 bacterial host (Ongvarrasopone *et al.,* 2007).

A different RNA replication system based on carrier state bacterial cells containing bacteriophage φ6 polymerase complex to produce large amounts of dsRNA up to 4.0 kb in length has been developed by Aalto *et al.* (2007; Figure 6). According to the authors, kilogram quantities of dsRNA can be made in industrial-scale bioreactors with this method. To develop this method, plasmid pLM1086 from *Pseudomonas syringae* (*P. syringae*) LM2691 (expressing T7 polymerase) was electroporated into *P. syringae* Cit7, yielding the host strain *P. syringae* Cit7 (pLM1086). Two additional plasmids (pLM991 and

[7] *Escherichia coli* (*E. coli*) Migula strain or *E. coli* strain HT115 was used in the study (Ongvarrasopone *et al.,* 2007). E. coli [HT115(DE3)] deficient in the enzyme that degrades dsRNAs can also be used to produce large quantities dsRNA (Zotti and Smagghe, 2015)

pPS9) were electroporated into the host cells. Plasmid pLM991 contains viral RdRP and other genes necessary for the formation of empty polymerase complexes (procapsids, PCs), and kanamycin resistance (kan). Plasmid pPS9 contains cDNA of the target gene (in this case eGFP, green fluorescent protein). In the host, ssRNAs are synthesized along with the PCs and are packaged inside the PCs. Upon packaging, an exact complementary strand is synthesized inside the PC particle by the viral RdRP. The resulting PCs containing dsRNA are harvested and purified.

It should be noted that manufacturing processes are likely to evolve since RNAi is an emerging technology.

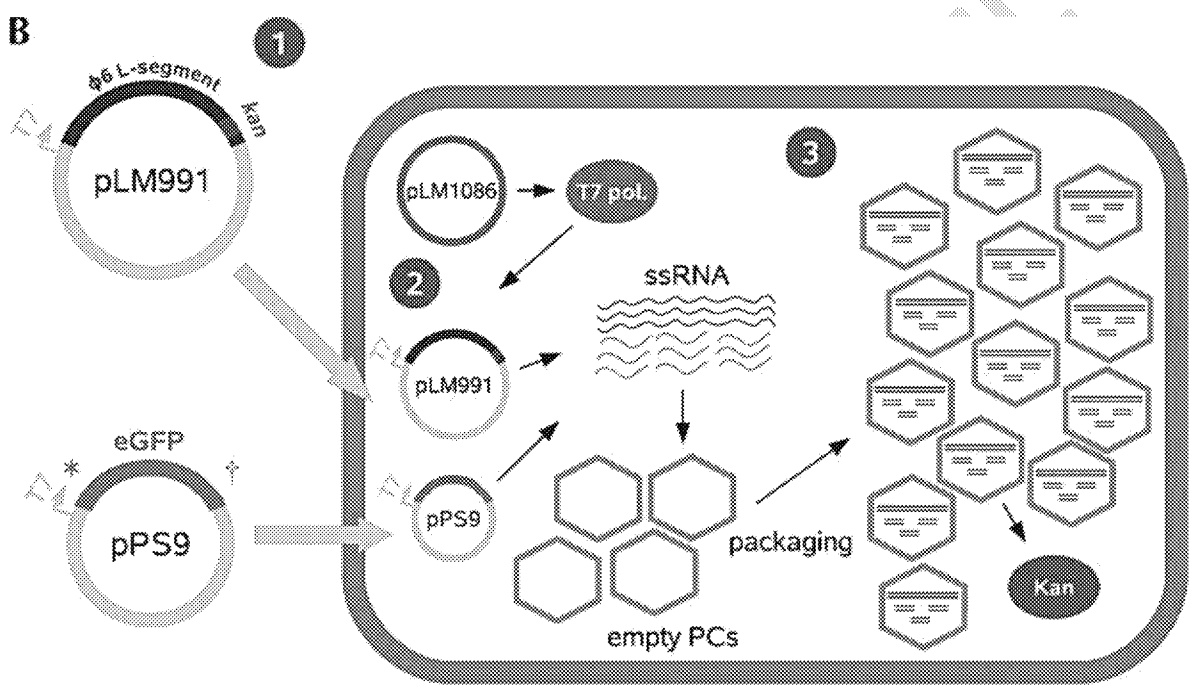


Figure 6. Production methods for long dsRNA (Aalto *et al.,* 2007). *In vivo* dsRNA production system utilizing bacteriophage φ6. The diagram depicts the formation of a stable carrier state relationship between φ6 and *P. syringae* Cit7 (pLM1086) host cells. ❶ Plasmids containing cDNA of the φ6 L_{kan} segment (pLM991) and the S_{eGFP} segment (egfp flanked by φ6 s-segment 5′-packaging (*) and 3′-replication (†) signals [pPS9]), placed under a T7 promoter, are electroporated into the host cells and maintained by kanamycin selection. ❷ The cells contain a plasmid (pLM1086) that constitutively expresses T7 RNA polymerase, transiently synthesizing ssRNA from the cDNA plasmids, which are non-replicative in *P. syringae*. The φ6 L_{kan} segment contains the viral RdRP and other genes necessary for the formation of empty polymerase complexes (PCs), and a kanamycin resistance (kan) gene. Packaging begins with S_{eGFP} ssRNA. ❸ Upon packaging, an exact complementary strand is synthesized inside the PC particle by the viral RdRP. Packaged capsids contain on average three copies of the S_{eGFP} segment and one copy of the φ6 L_{kan} segment.

If dsRNA is manufactured using genetically engineered microorganisms, procedures must be included in the manufacturing process to eliminate any viable microorganisms from the final product. These engineered microorganisms, if they remain viable, could significantly increase persistence of dsRNA and any potential off-target effects by growing in the environment or by spreading genes. Depending on the

species chosen, the engineered microorganisms could also directly affect non-target organisms through infectivity or the production of toxic metabolites. Additionally, antimicrobial resistance genes, which are often included in plasmid constructs, could be spread to naturally occurring environmental species.

Chapter 4: Formulation – Stabilizing dsRNAs

Very little is known about RNAi-based pest control formulations, but a number of formulation strategies have been developed in the past decade to address the bioavailability, delivery and toxicity potential of RNAi therapeutic products. From naked RNA formulations to liposomes, polymers and conjugates, all these formulation strategies attempt to limit RNA degradation in therapeutic products and could be adapted to exogenously applied products.

Chemical modifications

There are three main types of chemical modifications which are commonly used to render siRNAs suitable for therapeutic purposes:

1. Modifications to the phosphodiester backbone – this modification makes siRNA more resistant to nucleases and also improve biodistribution and cellular uptake.
2. Modification to the ribose 2'OH group – this modification increases thermostability and potency and reduces immunostimulation.
3. Modifications to the ribose ring and nucleoside base – this modification increases stability and knockdown strength by influencing base-pairing.

For a complete list of chemical modifications of siRNA, along with advantages and disadvantages, refer to Appendix III.

Conjugation

siRNA can be chemically bound to various biochemical components to increase cellular uptake.

1. Linking cholesterol to the 3' OH of the siRNA promotes uptake through receptor mediated endocytosis (Kim and Rossi, 2007; Rettig and Behlke, 2012).
2. Binding of ligands such as cationic lipids (i.e., transfection reagent lipofectamine), polymers and dendrimers to the siRNAs promotes uptake via adsorptive endocytosis.
3. Conjugation to cell penetrating peptides (CPPs) such as penetratin, transportin is another method through which endocytosis of the siRNAs is promoted. Receptor-specific peptides, hormones, antibodies and even vitamins can also be used.
4. Conjugation with bile acids or various long chain fatty acids can also promote cellular uptake.

Complexes

Packaging siRNAs in larger complexes is the best strategy to protect siRNA from degradation and clearance in the human body. Liposomes are the most popular delivery system to use in RNAi therapy. They are simple to synthesize and do not activate the immune system. However, they offer limited efficiency due to their neutral nature. When Whyard *et al.* (2009) fed four *Drosophila* species[8] dsRNA encapsulated in liposomes targeting the γ-tubulin gene, high mortalities were observed while none of the drosophilid species showed any evidence of RNAi when fed with non-encapsulated dsRNA. Stable nucleic acid-lipid particles (SNALPs) are a similar concept to liposomes; they have a positive charge that allows for more effective delivery with low toxicity.

The potential uses of nanotechnology have yielded the development of nanodispensers, nanogels, and nanocapsules (Chandrashekharaiah, 2015). Nanomaterials hold great promise regarding their application in plant protection due to their size-dependent qualities, high surface-to-volume ratio and unique optical properties. Chitosan particles have emerged as a valuable carrier for controlled delivery

[8] *Drosophila melanogaster, Drosophila sechellia, Drosophila yakuba,* and *Drosophila pseudoobscura*

of dsRNAs because of its proven biocompatibility, biodegradability, non-toxicity, and adsorption abilities (Kashyap *et al.,* 2015). Not only does chitosan provide a protective reservoir against degradation, it allows controlled release of the active ingredient (Kashyap *et al.,* 2015). Chitosan can easily make a complex with siRNA and forms nanoparticles (Kashyap *et al.,* 2015). A schematic representation of the interaction between the chitosan and the dsRNA is seen in Figure 7. Zhang X *et al*. (2010) successfully delivered dsRNA chitosan nanoparticles (100–200 nm) in stabilized form to mosquito larvae via feeding. In the study, two chitin synthase genes, AgCHS1and AgCHS2, were repressed by chitosan-dsRNA nanoparticles (dsAgCHS1-f1 and f2 or dsAgCHS2-f1 and f2) through third-instar larval feeding in *Anopheles gambiae*. The expressions of the genes were repressed by 48.4–63.4%, which suggested the potential use of nanoparticle-based RNAi technology for developing novel strategies for pest management.

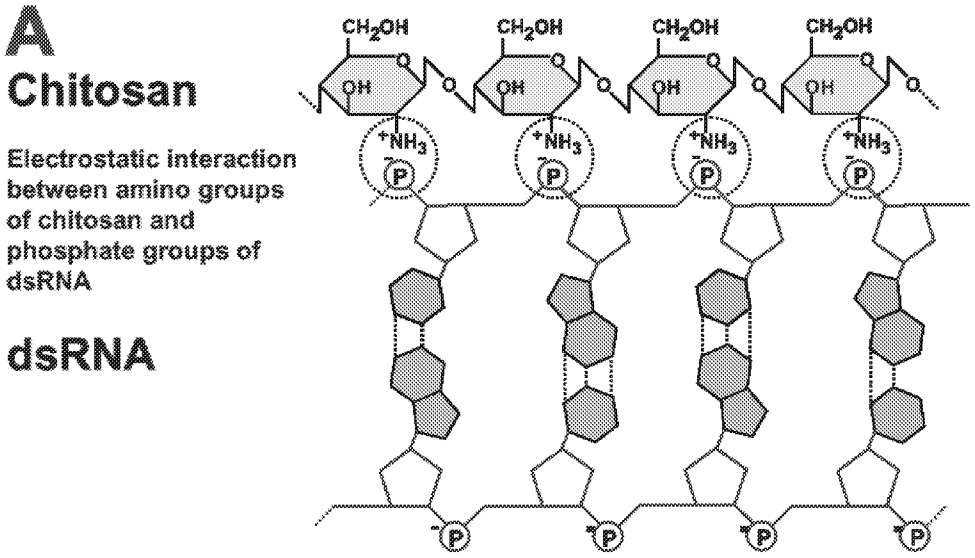


Figure 7. Schematic representation of electrostatic interactions between chitosan and dsRNA (Zhang X *et al.,* 2010).

In 2013, He *et al.* successfully utilized cationic core-shell fluorescent nanoparticles (FNPs; Figure 8) to deliver dsRNA in insects. In the study, chitinase-like gene, CHT10 was repressed by FNP-dsRNA through fifth instar larval feeding in Asian corn borers. Each larva was fed with 4 µg of dsRNA. After day 5, FNP/CHT10-dsRNA-fed larvae failed to molt.

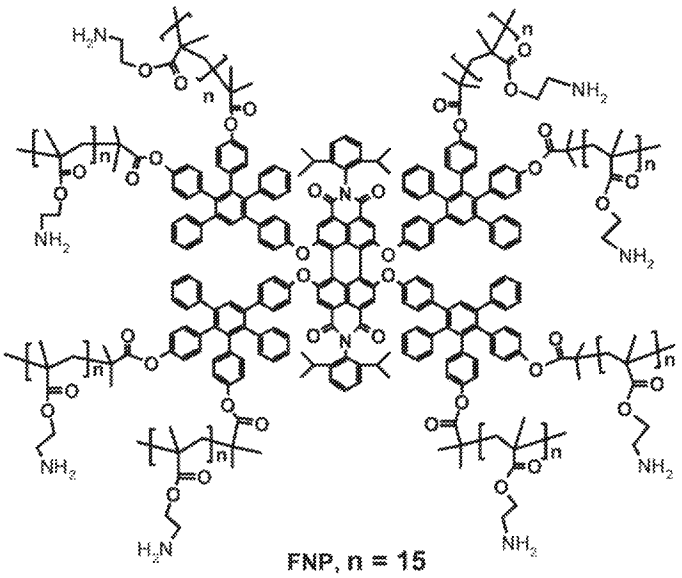


Figure 8. Structure of core-shell fluorescent nanoparticles (FNPs) containing functional amino groups. The FNPs in the study consisted of a fluorescent core of perylene-3,4,9,10-tetracarboxydiimide chromophore (PDI) in the center and polymer shells terminating with multiple amino groups.

Das *et al.* (2015) found that carbon quantum dot (CQD) was the most efficient carrier for dsRNA retention, and delivery compared to chitosan and silica complexes when used to target SNF7 and SRC in *Aedes aegypti* larvae.

Formulants/Inerts

A formulant is any substance or group of substances other than the active ingredient that is intentionally added to a pest control product to improve its physical characteristics (e.g., sprayability, solubility, spreadability and stability)[9]. Formulants such as wetting agent, dispersing agent, preservatives, soil conditioners etc., are expected to be present in the EPs. Additional formulants may be added to limit dsRNA degradation and improve bioavailability and delivery.

[9] Definition from Pest Management Regulatory Agency Formulants Policy and Implementation Guidance Document (2006) [HYPERLINK "http://www.hc-sc.gc.ca/cps-spc/pubs/pest/_pol-guide/dir2006-02/index-eng.php"]

Chapter 5: Persistence and Bioavailability of RNAs

5.1 Environmental Fate of RNAs

Environmental fate provides an indication of what happens to a pesticide once it enters the environment, as well as likely exposure levels for non-target organisms. The distribution and fate of exogenously applied dsRNA within the environment will likely depend on number of factors as follows:

1. dsRNA modifications
2. Presence of viable genetically engineered microorganisms;
3. Use pattern;
4. Offsite movement; and
5. Horizontal transfer of dsRNA.

dsRNA Modifications

Modifications used to stabilize dsRNAs (Chapter 4) are likely to increase the persistence of the dsRNA in the environment. This increase could lead to increased non-target exposure, potentially increasing the chances of unwanted effects caused by the dsRNA.

Presence of viable microorganisms

If viable microorganisms used for the production of dsRNAs are present in the EP (Chapter 3), in theory, they can continuously generate dsRNAs, leading to increased persistence in the environment.

Use Pattern

The use pattern, i.e., method of application, frequency of application and application rate, has a direct impact on environmental exposure. Environmental and non-target exposure increase with increasing application rates and/or frequency of applications. However, Monsanto (Submission of Comments, 2014) stated that the application rates are expected to be very low (i.e., grams per acre amounts).

Weather conditions at the time of application, such as air temperature and humidity, may affect the chemical volatility of the product (Damalas and Eleftherohorinos, 2011). When applied during increased wind, considerable spray drift exposure is likely to occur. The amount of pesticide that is lost from the target area and the distance the pesticide moves will increase as wind velocity increases. In addition, low relative humidity and high temperature will cause more rapid evaporation of spray droplets between the spray nozzle and the target than high relative humidity and low temperature.

The method of application is closely associated to the formulation type. Usually, localized treatments are often done with ground equipment, whereas the broad-scale treatments are usually done with aircraft (Damalas and Eleftherohorinos, 2011). Generally treatments with ground equipment minimize drift to non-target areas.

Off-site movement of applied dsRNA

The site of application influences the pesticides' potential for distribution. A pesticide applied directly to the soil may be transported via runoff (Briggs, 1992). For many pesticides, they are applied to cultivated crops; therefore contamination of non-target organisms is likely to occur in the fields rather than a forest system. With forestry application, the canopy can screen out most of the aerial spray, except for water-soluble pesticides, where the spray can be leached into water following precipitation (Briggs, 1992). Fat-soluble and persistent pesticides have some mobility via organic matter and are seldom

confined to the site of application. Application drift is dependent on wind and droplet sizes. The size of the area to be treated is also an important factor.

Offsite movement of dsRNA from the treatment site may occur through surface runoff from foliage and soil following a precipitation event; infiltration into the soil and movement into groundwater; and spray drift. Plant tissue and pollen movement may also be a factor if exogenously applied dsRNA is taken up and amplified[10] by RdRP within treated plant tissues/pollen, or if dsRNA was applied during anthesis (EPA White Paper, 2013). The amount and the distance moved will depend on the characteristics of the pollen (e.g., morphology, weight) and the mechanism relied upon for pollination (e.g., wind, pollinators, self-pollination) (EPA White Paper, 2013). Pollinators such as honey bees, if present on plants at the time of application, may carry dsRNA residues back to the hive. However, Monsanto (Submission of Comments, 2014) stated that several elements would limit off-site movement:

I. The anticipated low levels of exogenously applied dsRNAs for agricultural products,
II. Relatively small amounts of spray drift (1–5% of applied for ground or aerial applications), and
III. The rapid degradation of dsRNA in soil (degradation within 2 days).
However, it should be noted that San Miguel and Scott demonstrated that dsRNAs can survive more than 28 days on plants, and viral RNAs can survive 88 days in water (see Chapter 5 "*dsRNA degradation on plants*" for detail).

Horizontal transfer of dsRNA

Horizontal transfer of dsRNA is a possible concern with RNAi products if the microorganisms used for the production of dsRNA remain viable in the final products. Viable microorganisms may transfer dsRNA expressing plasmid to other organisms which may lead to increased non-target dsRNA exposure and persistence.

The EPA SAP (2014) mentioned that the potential for transferring dsRNA via ingested organisms may also occur. The uptake of dsRNA in herbivorous insects and non-target insects could impact predators. dsRNA transfer between soil and above ground organisms (e.g., rhizosphere microbes and invertebrates that can be consumed by above ground vertebrates and invertebrates) is also a possible concern. Garbian *et al.* (2012) was able to observe bidirectional transfer of dsRNA from honey bee to Varroa mites. In the study, dsRNA ingested by bees was transferred to Varroa mites and then from the mite to a Varroa-infested bee.

5.2 Stability of RNA in Environment

The structure of RNA is often required for its functionality and regulation in diverse cellular and regulatory processes. RNA is an intrinsically unstable molecule even in normal aqueous conditions regardless of the structural confirmation it assumes (EPA SAP, 2013). This instability is due to RNA's chemical nature, where the additional OH at the 2' position on the ribose sugar ring provides the destabilizing moiety through intra-hydrolytic degradation. Both acidic and basic conditions can drive intra-strand hydrolysis of RNA chains (Lilley, 2011). Additionally, numerous ribonucleases (RNases) are encoded by both prokaryotic and eukaryotic organisms that degrade all types of RNA molecules regardless of their structural conformation (Sorrentino, 2010).

[10] EPA (White Paper, 2013) noted the uncertainty regarding whether exogenously applied dsRNA will amplify within living plant tissue and if so, the unknown degree of amplification that will occur. This may result in higher levels of dsRNAs than at the time of application.

dsRNA degradation in soil

In the soil, persistence of dsRNA is likely affected by a number of abiotic (e.g., temperature, soil structure and type, UV-light) and biotic (microbial degradation) factors (EPA SAP, 2014). Binding of dsRNA to soil organic matter may decrease degradation, but such binding may also decrease availability to organisms (EPA SAP, 2014). Dubelman *et al.* (2014) conducted a study to determine the biodegradation potential of a DvSnf7 dsRNA transcript derived from a Monsanto GM maize product that confers resistance to corn rootworm. In the study, soil samples[11] were enriched with 7.5 µg of DvSnf7 RNA per gram of soil. Within approximately 2 days after application to soil, DvSnf7 RNA was degraded and biological activity was undetectable regardless of texture, pH, clay content and other soil differences (Figure 9).

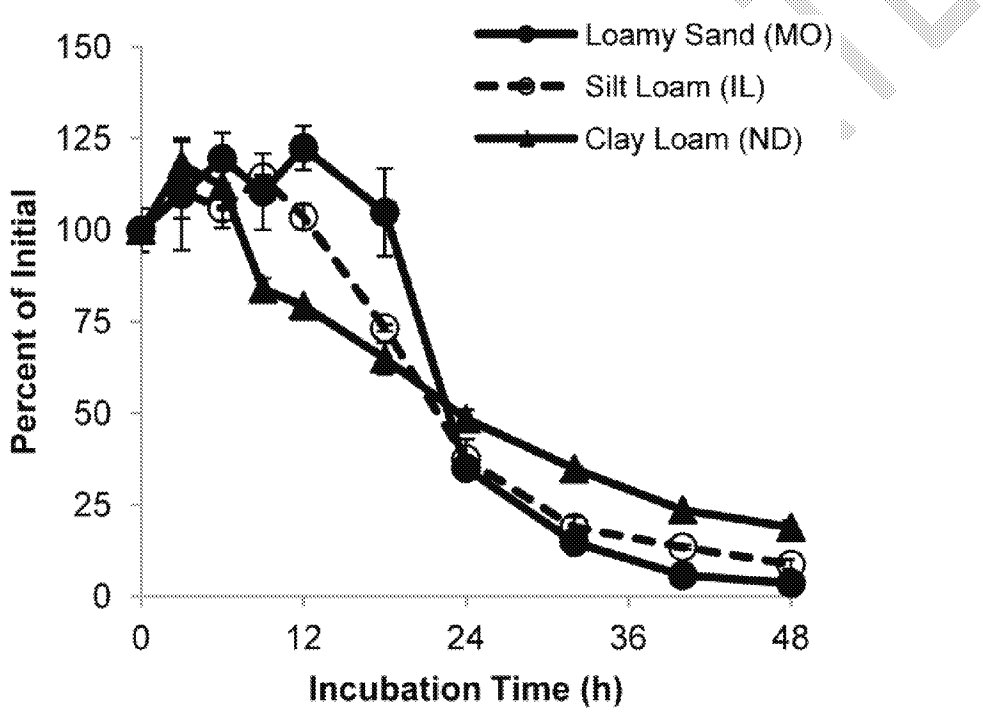


Figure 9. Degradation of DvSnf7 RNA degradation in three soil samples. DvSnf7 RNA was added in amounts of 7.5 µg per gram of soil. DvSnf7 RNA concentration was determined with a QuantiGene assay. Error bars represent one standard error of the mean (n = 2). Soil samples were from Missouri (MO), Illinois (IL), and North Dakota (ND).

dsRNA degradation on plants

San Miguel and Scott (2015) observed that foliar application of 5 µg Colorado potato beetle (CPB) actin-dsRNA/leaf protected potato plants for at least 28 days under greenhouse conditions. Second-instar CPBs placed on leaves treated with 5 µg of actin-dsRNA ceased feeding between 2 and 3 days. All CPB larvae did not reach fourth instar and resulted in low weight gain (~8% of the controls) and 98% mortality. As little as 1 µg of actin-dsRNA resulted in significant weight gain reduction compared to the control. However, no mortality or delay in development was observed at this concentration.

[11] Soil samples (States sampled from): Silt Loam (Illinois - IL), Loamy Sand (Missouri - MO), and Clay Loam (North Dakota - ND).

The study also tested the effects of rain on the activity of the applied dsRNAs. It was found that the dsRNA did not significantly wash off with rain simulation of 3 swirls, 3 seconds per swirl, through 250 mL of water when the dsRNAs was allowed to dry for 1 h on the leaves. The biological activity of the CPB actin-dsRNA was not significantly different between leaves that had been rinsed or not rinsed.

The study further tested the likelihood of dsRNA degradation under UV exposure. After 2 hours exposure to 1500 µW/cm of 254 nm UV light, the 297 base-pair CPB actin dsRNA (applied thinly on a glass surface) lost its biological activity. However, as mentioned before, the biological activity of the dsRNAs on the plant surface was retained even after 28 days in the greenhouse which suggests that the dsRNA is more stable on the leaf surface than on the glass surface used for the UV stability studies. It was hypothesized that the variation is due to the fact that the spray may be protected by shade from tiny hairs on the leaf or perhaps the spray soaks into the leaf (Ramanujan, 2015).

The study also demonstrated that dsRNA in water can be taken up by leaves if their petioles were incubated in the solution. This method was effective in reducing CPB's effects on plants however, it was not as effective as application of dsRNA to leaf surface. Conversely, the dsRNA did not appear to move systemically after foliar application. Biological activity was not observed in the nearby untreated leaves suggesting that there was no movement of dsRNA from treated to untreated leaves or the movement to the untreated leaves was insufficient for activity detection.

dsRNA degradation in water

Seitz *et al.* (2011) noted that purified Norwalk virus RNA (extracted from Norwalk virus virions) persisted for 14 days in groundwater, tap water, and reagent-grade water. Tsai *et al.* (1995) observed that viral RNA extracted from poliovirus could not be detected by RT-PCR (reverse transcriptase- polymerase chain reaction) after two days of incubation in unfiltered seawater but in filter-sterilized seawater, detection was observed after 28 days of incubation. This result is similar to those of Limsawat and Ohgaki (1997) in which seeded Qß RNA in autoclaved wastewater and autoclaved Milli Q water was detectable up to 88 days; while seeded Qß RNA in raw domestic wastewater and filtered wastewater was not found after 30 and 60 minutes of incubation, respectfully. These results seem to show that liberated RNA in water could be degraded soon after being released from the virus capsids and the activities of microorganisms present in wastewater seem to be involved in the degradation of the RNA inoculated in the sample.

It should however be noted that the above RNAs are single-stranded. Double-stranded RNAs are known to be more resistant to common and ubiquitous endonucleases that cut single-stranded RNAs although double-stranded RNAs can be efficiently cleaved by the less abundant type III bacterial RNAses (Espinosa *et al.*, 2008).

5.3 Bioavailability to Non-Target Organisms

There are several factors that determine the bioavailability of dsRNA to an organism:

1. Exposure route
2. Formulation
3. Natural host barriers
4. Mechanism for dsRNAs uptake into the cell; and
5. Interspecies variations in RNAi machinery.

Exposure Route

As mentioned in Chapter 1, it is anticipated that exogenously applied dsRNA products can be applied using the same methods as traditional chemical pesticides, therefore multiple routes of exposure are possible.

Exposure via ingestion is likely to be the main exposure source for exogenously applied products. Direct ingestion via plant surface, incidental ingestion via soil or water, and indirect ingestion via predator/prey interactions are all likely ingestion exposure routes for non-target organisms. However there are physical and biochemical barriers (i.e., digestive system and import mechanism) that limit dsRNA exposure after oral ingestion.

If exogenously applied dsRNAs are amplified by RdRP in the pollens, respiratory exposure route via spray drift or aerosolization may occur. However, the European Food Safety Authority (EFSA, 2014) considered this route of exposure of limited relevance as pollens are limited in number and both pollen and agricultural dust tend to be large[12] particles that do not migrate to the small capillaries of the lungs. Petrick *et al.* justifies that pollen will be deposited in and cleared from the upper respiratory tract, resulting in secondary oral exposure rather than pulmonary exposure, therefore, conducting only oral toxicity study is sufficient (Authors' response to Letter to the Editor, 2015).

As for contact exposure, organismal movement through the treated area may lead to exposure via the dermal route; however Monsanto (Submission of Comments, 2014) mentioned that aspects of physical barriers to dermal contacts (e.g., cuticle, fur, exoskeleton or integument) may limit or negate dermal absorption. Organisms inhabiting in soil, sediment or aquatic environment may gain exposure via the integument. For fish, uptake via the gills depends on many factors such as physiochemical properties of the dsRNA, water flow rate across the gill, the aqueous stagnant surrounding the gill, the gill epithelium and the rate of blood flow through the gills are likely to affect dsRNA concentration (Monsanto Submission of Comments, 2014).

It should be noted that exposure alone isn't enough to induce RNAi. High enough exposure concentrations as well as accessibility of the dsRNAs to the target site are required for an effect to be observed. In the later part of this chapter, factors that may limit RNAi will be discussed, including potential barriers and the variation in mechanisms.

Formulations

The formulation of a product has a large effect on potential absorption (Brown and Ingianni, 2013). Formulation ingredients and strategies may be used to significantly alter the natural bioavailability of dsRNA (see Chapter 4 for details).

Natural host barriers

For orally ingested RNAs, it is difficult to establish effective doses for RNAi silencing. In the digestive tract, RNA is subject to both non-enzymatic and enzymatic degradation. In mammals, the breakdown begins with mastication and exposure to degradative enzymes in saliva, followed by further digestion in the stomach and gut (EPA SAP, 2014). Pancreatic and intestinal nucleases and enzymes eventually metabolize RNA to mono-nucleotides and subsequent nucleosides and bases (Carver and Walker, 1995;

[12] Pollen is in the 90 – 100 µm size range, in contrast with respirable particles that are 10 µm (Source Authors' response to Letter to the Editor, 2015 [HYPERLINK "http://www.sciencedirect.com/science/article/pii/S0273230015000239"])

Hoerter *et al*., 2011; Rehman *et al*., 2011; Sorrentino *et al*., 2003). In addition to the RNases encoded within the genome, there are likely numerous others RNAses provided by the collection of microorganisms that colonize the gastrointestinal tract (EPA SAP, 2014). If the RNA avoids all the degradation processes, uptake of short RNA sequences in humans is predicted to be limited to the upper small intestine (Carver and Walker, 1995). Due to its size and charge, diffusion across cell membranes is difficult for RNA.

In insects, the first barrier encountered by ingested items is the midgut peritrophic matrix (PM), which is a chitin and glycoprotein layer that prevents large molecules and toxins from entering into midgut cells (Hegedus *et al.,* 2009). It has been shown that disruption of PM structure improves midgut permeability and causes adverse effects on insects (Barbehenn, 2001) and this disruption can be brought upon by cysteine proteases (Pechan *et al.,* 2002). In contrast to mammals, some insects have high levels of cysteine proteases in the gut (e.g., some coleopterans) (EPA SAP, 2014). Mao *et al.* (2013) showed that cysteine proteases were able to enhance the ingestion-mediated RNAi of insects. In the study, 3rd instar cotton bollworms larvae were fed an artificial diet supplemented with plant cysteine proteases, GhCP1 and AtCP2, and *E. coli* cells for 2 days. The larvae were then transferred to leaves of transgenic *Arabidopsis* plants expressing the dsRNA against the bollworm P450 gene CYP6AE14. While the transcript level of CYP6AE14 was moderately decreased in the control group, a stronger decrease of CYP6AE14 expression occurred in the larvae pre-treated with His-GhCP1 or His-AtCP2. Similar results were obtained with the His-tag purified fusion proteins of GhCP1 and AtCP2.

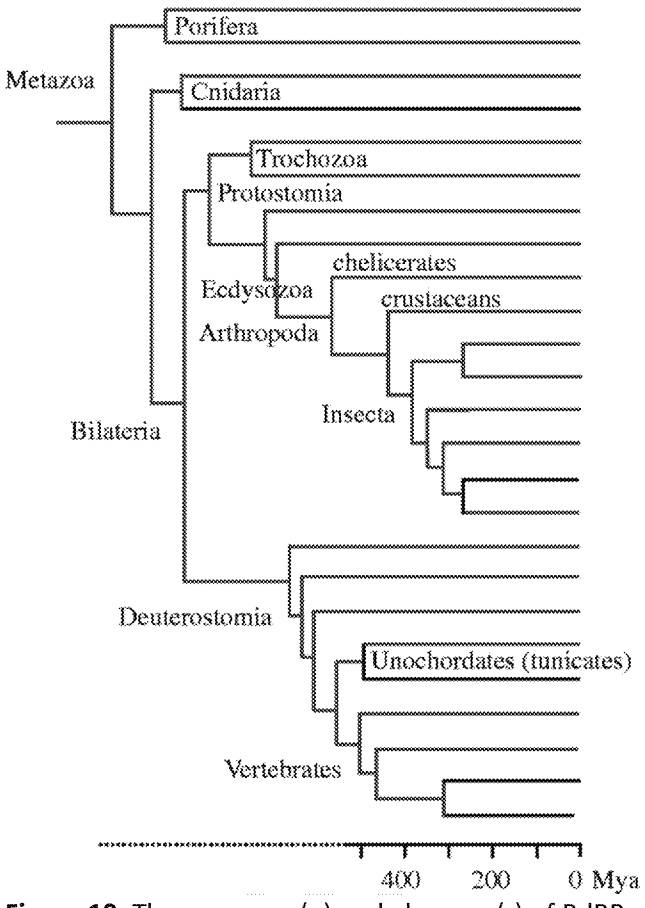
In mammals, there have been reports of miRNAs in human and bovine milk to be resistant to RNases (Admyre *et al*., 2007; Hata *et al*., 2010; Lasser *et al*., 2011; Zhou Q *et al*., 2012). These miRNAs appear to be resistant based on their incorporation into extracellular vesicles. Although there has been speculation as to the biological effects of these milk miRNAs, direct demonstration of such effects or transfer of RNA to the infant have not been done (EPA SAP, 2014).

Mechanism for dsRNAs uptake into the cell

Should significant quantities of ingested dsRNAs be absorbed across the GI tract and undergo distribution to tissues, Petrick *et al.* (2013) noted that in order to affect gene expression these molecules must: (1) cross cellular membranes; (2) escape from early endosomes to enter the cytoplasm; and (3) avoid degradation by nucleases found within lysosomes.

As described in Chapter 2.2, certain organisms have non-cell autonomous RNAi in the form of environmental or systemic RNAi. These two mechanisms allow the interfering effect to take place in tissues/cells different from the location of application or production of the dsRNA. The uptake and spread of dsRNA in nematodes was linked to two proteins: SID-1 and SID-2. Dietary uptake of dsRNA in *C. elegans* occurred when the dsRNA was recognized by the transmembrane protein, SID-2, in the environment of the gut (McEwan *et al*., 2012; Winston *et al*., 2007). Binding of the dsRNAs to SID-2 leads to endocytosis, followed by import across the cell membrane via a channel protein known as SID-1 (Hunter *et al*., 2006). Gene homologs of SID-1 were found in many organisms (Figure 10). Expressed sequence tag (EST) analysis indicated the widespread expression of SID-1-like genes in vertebrates, insects, parasites, and plants. In some organisms, such as *Drosophila* spp. and *Anopheles gambiae*, the lack of SID-1-like genes seemed to correlate with the apparent lack of a systemic RNAi response (Ren *et al*., 2011). A human ortholog of SID-1, SIDT1 was reported to facilitate uptake of cholesterol-modified siRNA (Wolfrum *et al.,* 2007) or contact-dependent transfer of human miR-21 between cultured cancers cells (Elhassan *et al.,* 2012). However, SID-1 is not essential for systemic uptake of dsRNA in certain insects as in the silkmoth *Bombyx mori* (Linnaeus), systemic RNAi was very difficult to achieve in spite of

three SID orthologue genes (Zotti and Smagghe, 2015). Contrary to SID-1, SID-2 gene is poorly conserved across organisms (EPA SAP, 2014). SID-2-dependent transport requires an acidic extracellular pH that is comparable to the conditions in the intestinal lumen and preferentially allows import of dsRNA of 50 nucleotides or longer (McEwan *et al.*, 2012). Alternative pathways that seem to aid in the uptake of dsRNAs have been identified in other organisms that lack functional SID-2, e.g., receptor-initiated endocytosis and scavenger receptors.



	RdRp	SIDs	Dicers
Oscarella	+	+	+
Reniera	–	3	2
Nematostella / coral	+	3	2
Hydra	+	1	2
mollusc	–	?	1
annelid	–	1	1
platyhelminthe	–	2	1
nematode	+	2+	1
tick	+	+	1
Daphnia	–	3	3
louse	–	1	2
aphid	–	1	2
beetle	–	3	2
honeybee	–	1	2
fly	–	–	2
moth	–	3	2
echinoderm	–	1	1
hemichordate	+	1	1
cephalochordate	+	3	1
Oikopleura	+	–	1
Ciona	–	–	1
lamprey	–	1	1
fish	–	1	1
birds	–	2	1
mammals	–	2	1

Figure 10. The presence (+) and absence (-) of RdRP and the number of SID and Dicer family members in different organisms (Obbard *et al.*, 2009).

Receptor-mediated endocytosis was first discovered in *Drosophila melanogaster* (*D. melanogaster*), where the clathrin heavy chain gene, a component of the endocytosis machinery, was identified (Ulvila *et al.*, 2006). Recent studies demonstrated that inhibition of the clathrin-dependent endocytosis pathway significantly reduces cellular uptake of dsRNA and suppresses RNAi (Wynant *et al.*, 2014; Xiao *et al.*, 2015). In the Wynant study, vacuolar H-ATPase 16 (*vha16*[13]) and clathrin heavy chain (*clath*[14]) genes were silenced in *Schistocerca gregaria*. To measure the effect of silencing *vha16*, the potency of t*alpha-tubulin 1a* (*tubu*) was used as a marker for uptake potency. Silencing of the *tubu* transcript was significantly less potent when *vha16* was down-regulated. To measure the effect of silencing *clath*, the potency of the g*lyceraldehyde 3-phosphate dehydrogenese (gapdh)* was used as a marker for uptake

[13] Mediates formation of coated vesicles
[14] Mediates lysosomal acidification

potency. Silencing of *gapdh* was significantly less robust when *clath* was downregulated. In Xiao's experiment (2015), four key genes relating to the clathrin heavy chain were silenced: TcChc, TcAP50, TcVhaSFD and TcRab7. When 16 day-old red flour beetle larvae were injected with each of the four dsRNAs, lethal giant larva (dsTcLgl) RNAi effects were significantly less potent. Thus, the pre-injections of all four dsRNAs targeting clathrin-dependent endocytosis significantly diminished RNAi of TcLgl in the larvae.

Scavenger receptors (SRs) are a group of structurally unrelated molecules known to mediate the endocytosis of certain polyanionic ligands, including nucleic acids (Ulvila *et al.*, 2006). In *D. melanogaster*, the scavenger receptors Class C scavenger receptor (SR-CI) and epidermal growth factor repeat-containing scavenger receptor (EATER) have important roles in uptake of dsRNA (Ulvila *et al.*, 2006). RNAi targeting the SR-CI and EATER led to a significant decrease (>90%) in the endocytosis of dsRNA fragments (500 bp) in *Drosophila* S2 cells. Similarly, Wynant *et al.* (2014) injected SR inhibitors, polyinosine (poly(I)) and dextran sulphate (DS), into the body cavity of the desert locusts and observed significant inhibition of *tubu*.

In mammals, although SRs uptake dsRNA, they trigger the interferon pathway rather than induce RNAi. Limmon *et al.* (2008) observed subsequent signaling and inflammatory cytokine and chemokine expression after extracellular dsRNA was recognized and internalized by scavenger class-A receptor (SR-A). Dieudonne *et al.* (2012) observed SRs such as LOX-1[15] and SR-B1[16] induce the activation of bronchial epithelial cells (BEC[17]) and participate in the internalization of maleylated ovalbumin (mOVA[18]).

Interspecies variations in RNAi machinery

RNAi is hybridization-dependent and thus occurs in a sequence-specific manner. It has been observed that a single base mismatch within the seed region of the siRNA may eliminate detectable siRNA-mediated silencing of the target (Amarzguioui *et al.*, 2003 and Du *et al.*, 2005). This is also true for sequences outside of the seed region as they are required for efficient target suppression by siRNAs. Amarzguioui *et al.* (2003) found that mutations in the middle of the siRNAs impaired silencing activity by a reduction of 20–30%. Furthermore, studies have shown that a contiguous sequence of ≥21 nucleotides is required to observe biological activity in a sensitive insect such as WCR and CPB. Baum *et al.* (2007) performed experiments based on the ingestion of heterospecific (different species) dsRNA that targeted V-ATPase subunits A and E and observed mortality in both WCR and CPB. This observation was due to the presence of 21-nucleotide shared sequences over the targeted portion of the V-ATPase gene for the two species. Bachman *et al.* (2013) repeated this study using Snf7 orthologs which did not have 21-nucleotide shared sequences and did not observe activity in either WCR or CPB when treated with the heterospecific ortholog.

However, even with correct binding for activity, gene silencing might not be detectible as there appears to be a threshold for RNAi. Cell culture studies indicate that at least 100 copies of siRNA molecules are required to induce RNAi in targeted mammalian cells (Brown *et al.*, 2007). Therefore, there may be environmental exposure levels, below which, no effects will occur (EPA SAP, 2013). There also seems to

[15] Lectin-like oxidised LDL receptor-1 is a class E SR which is a type II membrane glycoprotein that includes a type C lectin domain (Dieudonne *et al.*, 2012).
[16] Class B SR that are type II glycoproteins with a multiple transmembrane domain and in the extracellular domain, a loop maintained by di-sulfur links (Dieudonne *et al.*, 2012).
[17] Bronchial epithelial cells (BEC) regulate inflammatory and immune responses in the lung (Dieudonne *et al.*, 2012)
[18] Maleylated ovalbumin modulates the inflammatory response triggered by dsRNA (Dieudonne *et al.*, 2012)

be optimum concentrations of dsRNA required for gene silencing specific to each target gene and organism, such that exceeding this optimum may not necessarily result in additional gene silencing. San Miguel and Scott (2015) observed that concentrations greater than 5 µg of actin-dsRNA/leaf did not provide any significantly greater effects on CPBs.

In plants, nematodes and fungi, the RNAi silencing signal can be systemically amplified through the production of secondary siRNAs by RdRPs (Figure 11) (Vazquez and Hohn, 2013). This mechanism allows very low copy numbers of imported dsRNA to generate a robust RNAi response in any organism that possesses RdRP(s). In plants, siRNA signal from a source could be diluted over 10–15 cells, however, production of secondary siRNAs can extend silencing beyond the limited silencing zone (Nazim Uddin and Kim, 2013). RDR6 and SDE3 have been found to be key factors in amplifying secondary mobile signals (Nazim Uddin and Kim, 2013). Mammals and insects do not appear to have RdRP-mediated RNAi amplification (Gordon and Waterhouse, 2007), however, similar pathways may be present.

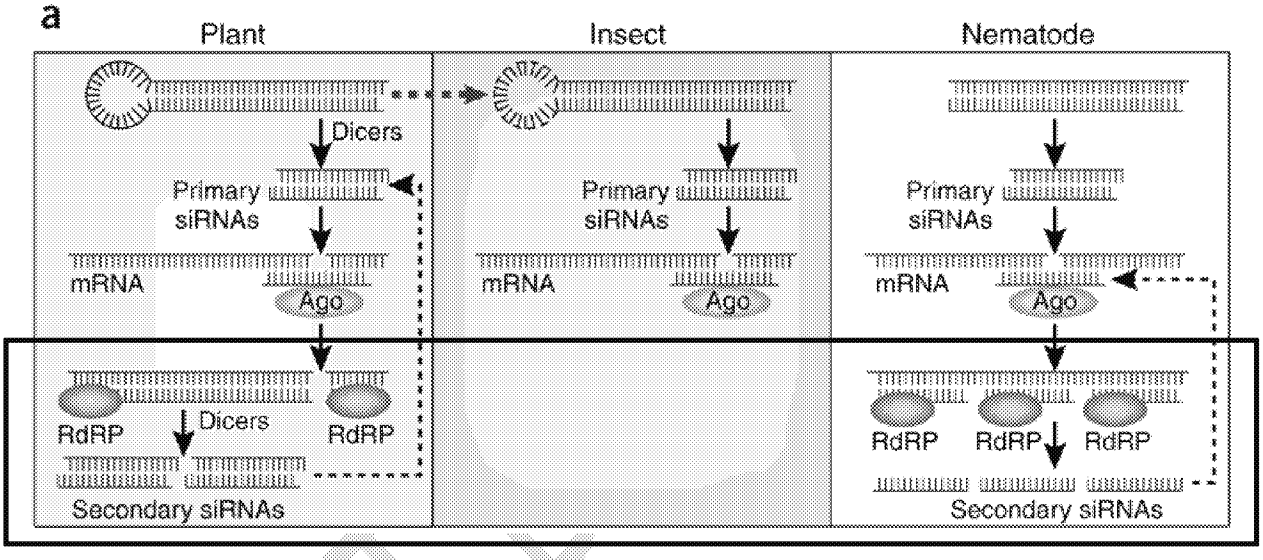


Figure 11. Comparison of known RNAi pathways in plants, insects and nematodes (Gordon and Waterhouse, 2007). The silencing amplification circuits that produce secondary siRNAs in both plants and nematodes are driven by RNA-dependent RNA Polymerases (RdRPs). In plants, RdRPs use the cleaved mRNA fragments as templates to synthesize long dsRNA; the dsRNA is then diced into secondary siRNAs. Nematode secondary siRNAs are produced by transcription rather than by dicing. (Ghildiyal and Zamore, 2009)

Silencing in different organisms

The variable effects of RNAi between mammals and insects are attributed to the wide range of gut pH; diet composition and feeding practices; conservation and function of RNA receptors and transmembrane channels; and activity of RNAses in digestive fluids and hemolymph (EPA SAP, 2014). Even among insects, RNAi silencing effects differ. Many members of the *Isoptera, Dictyoptera, Hemiptera, Orthoptera* and *Coleoptera* seemed to be highly responsive toward dsRNAs (Katoch *et al.*, 2013) while *Lepidoptera* and *Diptera* had demonstrated variable sensitivity to ingested dsRNA and high concentrations were required to elicit a response (Huvenne and Smagghe, 2010; Terenius *et al.*, 2011; Katoch *et al.*, 2013). In *Bicyclus anynana, Chrysodeixis includens* and *Spodoptera littoralis*, high doses of dsRNA (more than 1 mg/mg of tissue) did not result in any silencing effects while less than 10 ng per mg tissue was needed to induce silencing in *Hyalophora cecropia, Antheraea pernyi* and *Manduca sexta* (*M.*

sexta) (Terenius *et al.,* 2011). In *M. sexta*, and *Blattella germanica*, the difference in persistence of dsRNA is hypothesized to be mainly due to a nuclease in *M. sexta*'s hemolymph plasma (Garbutt *et al.,* 2013). A hypothesis regarding why certain insect orders are more susceptible to RNAi is due to the evolutionary selection pressure by baculoviruses (Heckel, 2015; Zotti and Smagghe, 2015). Many viruses were found in *Lepidoptera* following a search of the Ecological Database of the World's Insect Pathogens (Zotti and Smagghe, 2015), therefore it seems *Lepidoptera* spp. may have evolved defense mechanisms to generate more nucleases in the plasma and/or develop mechanisms to block the uptake of RNAs (Heckel, 2015).

Not only does silencing effects vary between insect orders, they also vary between species. Chu *et al.* (2014) observed that the silencing effects of dsRNA targeting DvRS5 (a cysteine proteases gene) varied between population of WCR. Three WCR populations exhibiting different levels of gut cysteine protease activity, tolerance of soybean herbivory, and immune gene expression were tested. Two populations were collected from crop rotation-resistant (RR) problem areas and one population was collected from a location where RR was not observed. Furthermore, silencing effects also seemed to vary due to life stage of the insect. Guo *et al.* (2015) found that dsRNA targeting S-adenosyl-L-homocysteine hydrolase decreased the target gene expression in an instar-dependent manner. Moreover, silencing effects vary due to different target site. For example, in *Lepidoptera*, the genes involved in immunity are the most likely to be susceptible to dsRNA-induced RNAi, whereas the genes expressed in the gut, salivary glands and gnathal appendages are the most likely to be susceptible in hemipteran species (Terenius *et al.*, 2011).

Successful RNAi in other vertebrates such as fish, reptiles, and birds has only been achieved with cell lines and/or embryos and has required the use of transfection agents, direct injection, electroporation, or other invasive techniques (Schyth, 2008; Sifuentes-Romero *et al.*, 2011; and Ubuka *et al.*, 2012). For plants, theoretically the cuticle in plants represents a significant barrier (Yeats and Rose, 2013), making unfacilitated dsRNA unlikely to penetrate the surface (Monsanto Submission of Comments, 2014). San Miguel and Scott (2015) demonstrated that dsRNA in water can be taken up the petioles and was effective in producing RNAi effects. The authors also showed that dsRNA did not move systemically after foliar application.

Chapter 6: Potential Hazards Associated with Manufacturing Process

Mass production of dsRNAs is likely to involve microorganisms. As mentioned in Chapter 3, dsRNA can be produced in bacterial cells by utilizing bacteria's RNA polymerases and fast production rate or by utilizing carrier state bacterial cells containing bacteriophage φ6 polymerase complex. The following potential hazards associated with the manufacturing process were identified:

Genetically Engineered Microorganisms (GEM)

Genetic engineering is the process of manually adding new DNA to an organism. The goal is to add one or more new traits that are not found in the organism. In the dsRNA production process, cDNA to the target gene is inserted into a vector which will be expressed in a microbial host. Potential unintended effects caused by genetic engineering may include the following:

I. Effects from Host Organisms
   a. Production of microbial/viral contaminants, microbial toxins, allergens and other metabolic products that pose a hazard to human and environmental health due to the expression of residual vector, host or adjacent host genetic material, not directly related to the intended function;
   b. Generation of unwanted infectious effects arising from the utilization of infectious virus;
   c. Expression of disease, carcinogens, mutagens and reactivation of dormant viruses due to horizontal transfers of related genes; and
   d. Continuous production of dsRNAs due to viable microorganisms in the EP. This may lead to increased persistence of the dsRNA along with increased exposure to non-target organisms. Furthermore, viable microorganisms in the EP may transfer plasmid/genes to other organisms thereby further increasing the persistence of the dsRNA and the chance of non-target effects.

II. Effects from the Transformation Process
   a. Incorrect trait expression and/or inconsistent inheritance due to instability of inserted gene; and
   b. Antibiotic resistance or related effects due to the use of antibiotic resistance or other markers of clinical or veterinary important.

Unintended Ingredients

In the manufacturing process, occurring impurities, contaminants or extraneous materials (such as the ones listed below) may pose potential hazards to organismal health and the environment.

a) Chemical reaction by-products;
b) Fermentation residues;
c) Materials impurities; and
d) Mutant, or alternate forms of the microorganism due to rearrangement of the plasmid during the fermentation process.

Formulants

As discussed in Chapter 4, a formulant is any substance or group of substances other than the active ingredient that is intentionally added to a pest control product to improve its physical characteristics (e.g., sprayability, solubility, spreadability and stability). Formulants are likely to be added to

exogenously applied dsRNA products which may pose potential organismal health and environmental hazards.

A potential delivery system for exogenously applied dsRNAs is nanoparticles. In RNAi therapy, a variety of natural and synthetic nanocarriers, including liposomes, micelles, exosomes, synthetic organic polymers, and inorganic materials have been developed for siRNA delivery and some of them have entered clinical evaluation (Shen *et al.,* 2012). Using such delivery system brings forth various uncertainties and unknowns; and whether chronic exposure leads to sufficient particle accumulation to trigger any RNAi response is unclear (Howard, 2012; Poland, 2012).

Chapter 7: Potential Health Hazards

7.1 Occupational, Bystander and Residential Exposure

Mammalian exposure to exogenously applied dsRNA is likely to be multi-routed. However, Monsanto (Submission of Comments, 2014) noted that even though direct contact is theoretically possible but because RNA is readily degradable in soil, there may be limited bioavailability of sufficient dsRNA to induce an RNAi effect with certain theoretical exposure routes. Conversely, San Miguel and Scott (2015) demonstrated that dsRNAs could last a minimum of 28 days in a greenhouse environment.

The EFSA (2014) believes that even if exogenously applied dsRNA is amplified within plant tissue/pollen, inhalation exposure can be considered as limited relevance, as pollen are limited in number and both pollen and agricultural dust tend to be large particles that do not migrate to the small capillaries of the lungs, and are not taken up effectively. Moreover, the aspect of physical barriers to dermal contacts (e.g., cuticle) may limit or negates dermal absorption (Monsanto Submission of Comments, 2014). It is also recognized that various factors (i.e., digestive system and uptake mechanism) may limit the exposure to dsRNA, refer to Chapter 5.3 for details. However, modifications used to stabilize dsRNAs in exogenously applied products (Chapter 4) to ensure sufficient residence time in/on the treated use site to permit the maximum desired pesticidal activity are likely to increase exposure to non-target organisms, possibly leading to unwanted effects.

7.2 Potential Health Hazards Associated with the RNAi End-Use Products

The risk of dsRNA pest control products has been largely debated. It has be suggested that due to the long history of consumption and the declaration of nucleic acids as Generally Recognized as Safe (GRAS) from the US Food and Drug Administration (FDA), RNA-based pest control products are of limited risks. However, counterarguments have been presented stating that above statements were not based on novel RNAs such as ones derived from modifications. Sequence homology is a key factor in determining off-target silencing. It has been shown that a minimum shared sequence length of 21 nucleotides is required for efficacy against WCR (Bachman *et al.,* 2013). Due to the small size of siRNAs (~21 nucleotides), the potential for siRNAs to be homologous to different gene sequence may be possible. Moreover, the process of RNAi can affect organisms in ways that goes beyond the effects of gene silencing. Introduction of exogenous dsRNAs may produce unintended immunostimulation, saturation of endogenous RNAi machinery and alterations of gut microflora.

7.2.1 Off-target Silencing

Off-target silencing is one of the primary concerns with the use of RNAi technology. Off-target gene suppression can occur when siRNAs hybridize with genes that have a high degree of sequence similarity to the intended target gene (Petrick *et al.,* 2013). Prior to 2012, dietary uptake of plant miRNAs in mammals was deemed nominal and non-specific, as dicer-produced siRNAs are well-defined and complex that off-target silencing are rare (Hannus *et al.,* 2014). However, a controversial study by Zhang L *et al.* (2012) reported that dietary plant miRNAs entered the mammalian bloodstream and regulated cholesterol metabolism. Four plant miRNAs were found in all samples, two of them occasionally reaching levels similar to those of abundant endogenous miRNAs. It was also reported that one plant miRNA, MiR168a, targeted an endogenous transcript involved in cholesterol metabolism, LDLRAP1 (a low-density lipoprotein), and raised circulating cholesterol counts. This particular study has generated

many follow-up studies by other scientists; none of which have successfully reproduced the particular experiment. Below is a list of studies on the dietary uptake of RNAs:

Evidence for bioavailability to mammals	Evidence against bioavailability to mammals
Zhang, L. *et al.,* 2012, study described above.	*"Our analysis suggests that plant miRNAs observed in some public animal sRNA datasets and our own insect feeding experiment sequence data may be artifactual due to sequencing methodology, and that accumulation of plant miRNAs via diet is not a common faculty among animals."* (Zhang Y *et al.,* 2012)
"We observed that a significant fraction of the circulating RNA appear to originate from exogenous species. […] Some of these RNAs are detected in intracellular complexes and may be able to influence cellular activities under in vitro conditions." (Wang K, 2012*)	*"Our results indicate that, even if some plant miRNAs appeared to amplify from nonhuman primate plasma, their levels were quite low and/or amplification was non-specific."* (Witwer, 2013)
"After drug treatment, the levels of a number of transcripts, both endogenous and exogenous RNAs, showed significant changes in plasma." (Wang K, 2013*)	*"In spite of […] ingestion, we find little evidence of significant steady-state expression of those miRNAs in recipient organisms (< 1 copy per cell in various organ tissues)."* (Snow *et al.,* 2013)
"Exogenous plant miRNAs were present in the sera, feces, and tissues of animals and these exogenous plant miRNAs were primarily acquired orally. MiR-172, the most highly enriched exogenous plant miRNA in B. oleracea*, was found in the stomach, intestine, serum, and feces of mice that were fed plant RNA extracts including miR-172. The amount of miR-172 that survived passage through the GI tract varied among individuals, with a maximum of 4.5% recovered at the stomach of one individual, and had a range of 0.05–4.5% in different organs. Furthermore, miR-172 was detected in the blood, spleen, liver, and kidney of mice."* (Liang *et al.,* 2014*)	*"Overall, our results show neither apparent uptake of ingested plant miRNAs by mice nor regulation of target protein levels in liver and plasma or phenotypic changes in mice from ingested plant miRNAs that would be indicative of target gene regulation after rice feeding."* (Dickinson *et al.,* 2013)
"Dietary milk-based microsomes appear to provide a mechanism for oral delivery into healthy consumers." (Baier *et al.,* 2014)	*"Plant miRNAs were not detected in our sequencing of human sperm cells, which was performed in the absence of any known sources of plant contamination."* (Tosar *et al.,* 2014)
"MIR2911, a honeysuckle (HS)-encoded atypical microRNA, […] is highly stable in HS decoction, and continuous drinking or gavage feeding of HS decoction leads to a significant elevation of the MIR2911 level in mouse peripheral blood and lung." (Zhou Z *et al.,* 2014)	A 28-day oral toxicity evaluation of siRNAs and long dsRNA targeting vacuolar ATPase in mice observed no treatment-related toxicity clinical effects[19]. NOAELs for 21-mer siRNAs and a 218 bp dsRNA were 48 and 64 mg/kg/day, respectively. Oral dsRNA exposure did not result in suppression

[19] Mortality, abnormalities, changes in body weight, organ weight, gross lesions or microscopic findings, signs of pain and distress and hematology parameters.

	of the mouse vATPase gene. *"The results of this study indicate that orally ingested dsRNAs, even those targeting a gene in the test species, do not produce adverse health effects in mammals."* (Petrick *et al.*, 2015)
"Our results suggest that tumor suppressor miRNAs designed to mimic small RNAs produced in plants were taken up by the digestive tract of $Apc^{Min/+}$ mice upon ingestion, as evidenced by their higher concentration in the miRNA-treated animals, and were functional, as evidenced by the reduction in tumor burden." (Mlotshwa, 2015)	

**showed miRNA can survive cooking and digestion and are bioavailable in humans and mice.*

It should be noted that most dietary studies conducted on uptake of dsRNAs are based on regular food and not based on transgenic plants or plants sprayed with exogenously applied dsRNA products.

Three critiques to the study conducted by Zhang L *et al.* (2012) have been summarized by Witwer and Hirischi (2014). First, the variability of the results was put into question. Witwer and Hirischi believed the large donor pool variability (MIR168a varied >2000 fold) in Zhang L *et al.* (2012) study was caused by the small sample size of the study and therefore, the results should not be reflective of the general public. If the variation was not due to the small sample size, technical variability[20] or batch effects[21] leading to false positives and significant variations between pools could be the explanation. Second, to fulfill the rapid increase of levels of MIR168a and decrease of target LDLRAP1 (50% in 3 hours[22]), serum LDLRAP1must have a short half-life (to experience >50% reduction in less than 3 hours) along with being able to double in less than 3 hours with 100% suppression of LDLRAP1 transcript. It is uncertain whether or not these factors were fulfilled in the study. Third, the relevance of the results was put into question. To exhibit the apparent regulation of LDLRAP1 of the study, a 55 kg human needs to eat 33kg of cooked rice per day. The activity of MIR168a in humans can be deemed as negligible.

Machinery factors that may contribute to off-target silencing:

Environmental and Systemic RNAi

The ability for environmental and/or systemic uptake of dsRNAs allows the gene interfering effect to take place in tissues/cells different from the location of application or production. Both mice and human genomes harbor two SID-1 homologs, SIDT1 and SIDT2. SIDT1 has demonstrated a role in the uptake of dsRNA by human cells. *In vitro* studies blocking or silencing SIDT1 resulted in a defect in the internalization of cholesterol-conjugated siRNA by human hepatocytes (Wolfrum, 2007) and the overexpression of human SIDT1 in pancreatic ductal adenocarcinoma cells enhanced the passive uptake of siRNAs (Duxbury *et al.*, 2005).

[20] Technical variability resulting from RNA extraction, sequencing, or library construction.

[21] Batch effects from collection, storage, purification, and experimental factors and/or contamination from oligonucleotide standards and non-dietary environmental plant matter.

[22] In the study, 3 hours after feeding, there was no significant difference in plasma or liver levels of MIR168a between mice fed regular chow or raw rice. By 6 hours, a 50% increase in serum and a two fold increase in liver were observed, accompanied by >50% decrease of putative target LDLRAP1.

Amplification Mechanisms

Cell culture studies indicated that at least 100 copies of siRNA molecules were needed to reach a targeted cell site to induce RNAi in mammalian cells (Brown *et al.*, 2007). Therefore, under a certain exposure level, silencing might not occur even with uptake of dsRNAs. Organisms with an amplification system will be able to generate a robust RNAi response from very low copy numbers of imported dsRNAs. RdRP-mediated RNAi amplification has not been identified in mammals; however, other mechanisms might be present.

The EPA Science Advisory Panel (SAP) concluded in 2014 that the available evidence supports the conclusion of no significant absorption of dsRNA in mammals and minimal likelihood of adverse effects, however, they stressed that data are lacking in this area and there are no published studies involving plants sprayed with exogenously applied dsRNA products.

7.2.2 Immunostimulation

Innate immune systems of higher organisms rely on pattern recognition proteins and other factors to identify potentially pathogenic invaders including foreign dsRNAs. The theoretical potential of plant RNA-stimulated innate responses in mammals is possible. Generally speaking, siRNAs are able to trigger mammalian endosomal immune cascades (e.g., Toll-like receptors (TLRs)[23]), or cytoplasmic pathways (e.g., RIG-1[24], Mda-5, PKR[25]) (Sioud, 2015). Immunostimulation appears to be sequence and structure-dependent, controlled by Toll-like receptors 7 and 8 (TLR7, TLR8) immune stimulatory RNA motifs and not by the length of the siRNA (Forsbach *et al.*, 2011). Zhou R *et al.* (2007) observed systemic inflammation and damage to organs including the gut when 5 µg/g weight of foreign RNA were injected into mice. It should be noted that the route of exposure in this study is unlikely for exogenously applied dsRNA products. Petrick *et al.,* (2015) observed inflammation in 1/8 male mice at the oral dose of 64 mg/kg/day with 218-bp dsRNA and 1/8 female mice at the oral dose of 48 mg/kg/day. Due to the high doses required to cause the deleterious effects, the EFSA (2014) and the EPA (SAP, 2014) stated that it is unlikely that novel siRNAs would cause an immune response.

7.2.3 Saturation of Machinery

Oversaturation of RNAi machinery as a result of introduction of exogenous dsRNA can disrupt regulation of gene expression and normal cell function (Katoch *et al.*, 2013). Saturation can also lead to reduced defenses against viral infection (Dillin, 2003). Essentially, there is a limited number of RISCs present within a cell, and if the augmented siRNAs saturate these complexes, then health and performance of the cell may be compromised (Kahn *et al.*, 2009).

Grimm *et al* (2006) hypothesized that the toxicity and mortality in wild-type C57/BL6 or FVB mice observed when a high dose (10^{12}) infusion of high short hairpin RNAs (shRNAs) was introduced, was

[23] Toll-like receptors (TLRs) are involved in the early immune recognition of invading pathogens. Stimulation of the TLR results in the initiation of signalling cascades which ultimately lead to the activation of immune cellular responses including the production of pro-inflammatory cytokines and interferons (Sioud, 2015).

[24] Retinoic-acid-inducible gene I (RIG-1) is a cytoplasmic sensor of viral RNA. Mice deficient for RIG-1 were found to be highly susceptible to viral infection (Sioud, 2015).

[25] dsRNA-dependent protein kinase (PKR) is a sensor for dsRNA recognition. Upon binding to dsRNA, PKR forms a homodimer resulting in its autophosphorylation and activation. Activated PKR phosphorylates a large number of substrates, particularly the translation initiation factor eIF-2α leading to translation arrest and induction of apoptosis, an essential step in antiviral resistance. PKR can also activate of the NF-κB signaling pathway via the phosphorylation of IKKβ (Sioud, 2015).

associated to competition of the miRNA components. Other studies also found that RNAi components may be saturable. Kahn *et al.* (2009) found that siRNAs concentrations from 4nM were able to saturate RISC components, while Grimm (2011) observed Exportin-5 and Argonaute proteins (especially AGO2) saturation when $5x10^{11}$ to $2x10^{12}$ copies of exogenous siRNAs were introduced to mice (Grimm, 2011). High copies of viral associated RNA (10^8 copies/cell) were also able to saturate the RNAi pathway (Andersson *et al.*, 2005). However, the EFSA (2014) and the EPA (SAP, 2014) stated that it is unlikely that under realistic exposure conditions, the dose would be sufficient enough to affect RNAi machinery.

7.2.4 Effects on Gut Microbiome

Bacteria and archaea have RNA-based regulatory systems but the machinery, RNA sequences, and binding behavior for these systems differ from those in eukaryotic systems (Rusk, 2012). In bacteria, the clusters of a regularly interspaced short palindromic repeat (CRISPR[26]) locus produces CRISPR RNA (crRNA) that guide CRISPR-associated (Cas) proteins to target foreign nucleic acid (Heidrich and Vogel, 2013). CRISPR interference (CRISPRi) can target transcription in bacteria and human cells (Larson *et al.*, 2013). It has said that CRISPRi is highly effective at gene silencing compared to RNAi (Taylor and Woodcock, 2015).

Currently, the effects of exogenous dsRNAs on the microbiome in the human and animal gut and the possible influence on their homeostasis are unknown (EPA SAP, 2014). In addition, there is no evidence that eukaryotic dsRNA is amplified by bacteria. However, Petrick stated that the potential hazards posed by the product on gut microbiome may be dismissed as bacteria uptake RNA from the environment as food through the CRISPR-CAS system, and do not uptake RNA resulting in an impact on gene expression.

7.3 Influence of Human Health Conditions and Vulnerable Populations

Inflammation associated with infectious and non-infectious GI tract disease, stress, and malnutrition, obesity and alcohol use can lead to a leaky GI tract that favours the uptake of dRNAs (Witwer and Hirschi, 2014). Individuals who manifest specific diseases (e.g., Crohn's, colitis, irritable bowel syndrome, etc.), or are immunocompromised, elderly, or are children may have compromised digestion or increased sensitivity to dsRNA exposure (EPA SAP, 2014).Digestion conditions, intestinal permeability, glomerular filtration, distribution, and persistence of exogenous RNA in the body may differ in these individuals such thatspecial considerations may need to be applied.

[26] CRISPRs consist of multiple copies of a short repeat sequence (typically 25 - 40 nucleotides) separated by similarly-sized variable sequences that are derived from invaders such as viruses and conjugative plasmids (Hale *et al.*, 2009; Sampson *et al.*, 2013).

Chapter 8: Potential Environmental Hazards

8.1 Potential Environmental Hazards Associated with RNAi End-Use Products

As mentioned in Chapter 5, there are various factors such as dsRNA modifications, use patterns etc. that contribute to the environmental fate of dsRNAs. The stability of dsRNA in soil appeared to be low as complete degradation was observed within approximately 2 days after application. However, dried-on spray on plant seemed to last a substantially long time (more than 28 days). In autoclaved wastewater, viral RNAs were shown to persist after months. The routes of exposure for non-target organisms seem to encompass ingestions, inhalation, and contact. Exposure to dsRNAs does not necessarily mean effects will be produced as natural host barriers such as the digestive system may lead to degradation of the dsRNAs before an effect can be generated. Other factors such as the presence or absence of dsRNA uptake mechanisms (i.e., SID proteins) may aid or inhibit RNAi effects.

Off-target silencing is one of many concerns with use of RNAi technology. Each organism's genome presents a unique set of potential off-target gene sequences. It has been noted by Qiu *et al.* (2005) that the likelihood of non-target binding increases with the size of the genome and therefore reduced hazard is posed with relatively small genomes. Another concern with RNAi technology is the potential activation of the immune system. Immunostimulation appears to be sequence and structure-dependent but not length dependent. The potential of oversaturation of RNAi machinery as a result of introduction of exogenous dsRNA is another potential hazard which can disrupt regulation of gene expression and normal cell function (Katoch *et al.*, 2013). Saturation can also lead to reduced defenses against viral infection (Dillin, 2003). Current information regarding RNAi in living organisms is limited to mammals and arthropods, and little work has focused on the barriers to uptake that exist in other organisms (EPA SAP, 2014). In Canada, the environmental risk assessment typically considers the hazards to arthropods, birds, wild mammals, fish, non-arthropod invertebrates, microorganisms, and plants. These taxa are considered sufficient representatives of the potential for risk to all non-target organisms for purposes of screening level risk assessments, though refined risk assessments may consider exposure at lower taxonomic levels.

8.1.1 Terrestrial Arthropods

RNAi has been observed in insects with varying degree of efficacy (a short discussion of it can be found in Chapter 5.3 "*Silencing in different organisms*"). Below are some studies involving RNAi via ingestion in terrestrial arthropods utilizing dsRNAs.

- Kim E *et al.* (2015) observed maximal insecticidal activity in *Spodoptera exigua* after ingestion of 350 ng dsRNA targeting SeINT via transformed *E.coli*.
- Kumar *et al.* (2009) observed 20%, 35% and 60% mortality of *Helicoverpa amigera* larvae fed with 25, 50 and 75 nM of AChEsiRNA coated leaves respectively.
- Wan *et al.* (2014) observed 35–55% silencing effects when 0.5M of dsRNA against LdRyR was ingested by CPB.
- CPB fed on foliage-soaked dsRNA targeting *Ldalt* for 3 days observed 71.1–79.5% *Ldalt* mRNA reduction, and 64.5–67.6% protein reduction (Wan *et al.*, 2015). The foliage was soaked in a 0.5µg/µL dsRNA solution.
- Yang *et al.* (2014) observed 77.9–81.8% suppression in *Sogatella furcifera* after ingestion of 0.5M dsRNA against SfRyR.

- Levine *et al.* (2015) observed that ingestion of 0.0031 µg/ml DvSNf7 dsRNAs resulted in 50% growth inhibition in SCR. The LC_{50} value for DvSnf7 was estimated to be 0.0071 µg/ml. Growth inhibition was evident after 3 days of feeding and reached ~80% after 12 days. Mortality was not evident until day 6; this was similar to WCR.
- Turner *et al.* (2006) targeted a pheromone binding protein in light brown apple moth third instar larvae via ingestion of dsRNA and observed a reduction in level of transcripts persisted for approximately 18 days.

Off-target Gene Silencing

Within Target Organisms

In insects, siRNAs off-target silencing within a target organism can be introduced via two mechanisms. The first method is the selection of the incorrect strand as the RISC complex discarded the wrong strand, making the passenger strand the guide strand. This will in turn silence genes complementary to the passenger strand (Kanasty *et al.*, 2012). The second method of off-target silencing is the imperfect binding of siRNAs to the 3'UTR. In this case, the siRNA acts as a miRNA and can reduce the expression of non-target genes (Deng *et al.*, 2014; Bramsen and Kjems, 2012).

In Non-Target Organisms

Sequence homology is a key factor in determining off-target silencing. Whyard *et al.* (2009) demonstrated that with a 21-nucleotide long siRNA, 19–21 continuous nucleotides must be homologous to induce RNAi silencing in insects. In four species of *Drosophila* (*D. melanogaster, D. sechellia, D. yakuba, and D. pseudoobscura*), with each species having 79–96% sequence similarity throughout the coding sequence of the target gene with the other species, RNAi cross-silencing did not occur in any species as no 19–21 nucleotide length of sequence was shared among the four species. Another study demonstrated that siRNAs originally intended to target WCR caused the silencing of other coleopterans such as potato colorado beetle and southern corn rootworm (SCR) even though WCR and SCR shared 83 and 79 % sequence identity in vATPase A and vATPase E region, respectively (Baum *et al.*, 2007). To test the potential off-target effects of DvSnf7, the dsRNA was tested on honey bee via dietary assay (Tan *et al.*, 2015). The honey bee larvae and adults were fed 10µL of 1µg/g (~11.3 ng/larva) of dsRNA. 100% of the larvae survived and the adult emergence day did not significantly differ from the control (15.6 ± 0.4 days for control; 15.5 ± 0.3 days for the dsRNA treatment group). The NOEL for the larvae was deemed as ≥11.3 ng/larva. As for the adult honey bees, after 14 days of continuous feeding, no significant differences were observed between the control and treatment group; the survival rates were 92.5±1.44%, 91.25±2.39% and 0% in the treatment, negative control and positive control respectfully. Therefore the NOEL for the adults bees were deemed as ≥1µg/g diet. As for bioinformatics analysis, the Snf7 240 nucleotide ortholog sequence only had 72.5% similarity, with no 21-nucleotide contiguous matches.

Machinery factors that may contribute to off-target silencing:

Environmental and Systemic RNAi

RNAi triggered by feeding and soaking has been demonstrated in a variety of arthropods including ticks, honey bee and WCR (Whangbo and Hunter, 2008). Cellular uptake of dsRNA may be assisted by transport proteins such as lipophorins in some insects (EPA SAP, 2014) and SID proteins seen in various insects (Obbard *et al.*, 2009) (refer to Chapter 2.2 for more information on SID proteins). However the presence of these systems is not sufficient in determining off-target silencing as observations in *Lepidoptera* indicate that environmental RNAi does not occur uniformly in insects. For example, oral dsRNA delivery leads to effective systemic gene silencing in *Epiphyas postvittana* larvae (Turner *et al.*,

2006) but not in *Spodoptera litura* (*S. litura*) (Rajagopal *et al.,* 2002). The failure to perform environmental RNAi in *S. litura* may be explained by physiological differences in the gut environment between species or by variations in feeding techniques or dsRNA amounts. In *D. melanogaster*, environmental RNAi seems to function under certain conditions; soaking *D. melanogaster* embryos in dsRNA solutions can initiate RNAi (Eaton *et al.,* 2002); however, RNAi in response to dsRNA feeding has not been reported in *D. melanogaster* larvae or adults.

Amplification Mechanisms

RdRP amplification pathways are not present in insects, except in ticks (EPA SAP, 2014; Whangbo and Hunter, 2008; Obbard *et al*., 2009). It is possible that other pathways are present.

An off-target process called transitive silencing may arise with RdRP amplification (Figure 12). Transitive silencing occurs when secondary siRNAs from RdRP amplification extends towards regions upstream and downstream of the initial target site (Senthil-Kumar and Mysore, 2011; Vazquez and Hohn, 2013). This may affect the specificity of silencing, and lead to amplification of less specific siRNAs, causing off-target silencing.

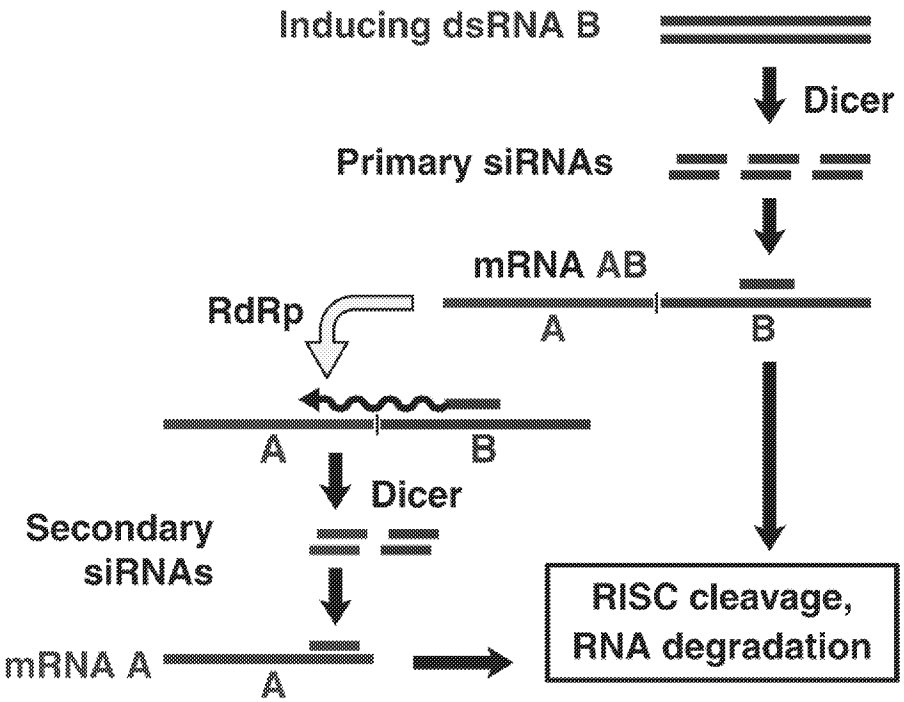


Figure 12. Transitive RNA silencing (Ahlquist, 2002). RdRP's action on intermediary mRNA AB, which extends the production of dsRNA towards regions downstream (A) of the initial target site (B), therefore generating off-target secondary siRNAs.

Immunostimulation

It is not clear how the immune systems of non-mammal organisms will react to an influx of small RNAs nor is it known how this immunostimulation will affect the fitness of non-target organisms (EPA SAP, 2014)

Saturation of Machinery

Similar RNAi pathway components are present in mammals and insects (Appendix I); saturation of Dicer, AGO2, and Exportin5 is theoretically possible (see Chapter 7.2 for dose details). However, the EFSA

(2014) does not consider saturation of the RNAi machinery of arthropods plausible under realistic exposure conditions.

8.1.2 Aquatic Arthropods

Successful induction of RNAi in crustaceans (*Peneaus monodon*) via ingestion has been achieved. White spot syndrome virus, is lethal for shrimp populations (Sánchez-Paz, A, 2010). Significant amount of *Penaeus monodon* was able to survive after bacterially expressed dsRNAs against white spot syndrome virus (WSSV) were orally administered to the shrimps. The dsRNAs were delivered in the form of: 1) pellet feed coated with inactivated bacteria containing overexpressed dsRNA or 2) pellet feed coated with VP28dsRNA–chitosan complex nanoparticles. After 30 days of feeding lead to 86% (utilizing method 1) and 37% (utilizing method 2) survival of the pre-treated shrimps (Sarathi *et al.,* 2008). 15 µg or three 2 µg injection of dsRNA against WSSV protected the shrimps for 28 days (Kumar, 2015).

8.1.3 Birds

Off-target Gene Silencing

Successful RNAi has been induced in birds. Ubuka *et al.* (2012) administrated by infusion 0.5 nmol of siRNAs against gonadotropin-inhibitory hormone (GnIH) precursor mRNA into the third ventricle of male and female white-crowned sparrows and observed reduced resting time, spontaneous production of complex vocalizations, and stimulated brief agonistic vocalizations. However, it should be noted that the exposure route described in the study is not expected to occur following the use of exogenously applied products.

Machinery factors that may contribute to off-target silencing:

Environmental and Systemic RNAi

SID-1 transport protein homologues have been identified in birds for potential dsRNAs uptake (Figure 10) (Obbard *et al.*, 2009).

Amplification Mechanisms

RdRP pathways are not present in birds (Obbard *et al.*, 2009); however, it is not known if other amplification pathways are present.

Immunostimulation

It is not clear how the immune systems of non-mammal organisms will react to an influx of small RNAs nor is it known how this immunostimulation will affect the fitness of non-target organisms (EPA SAP, 2014).

Saturation of Machinery

Dicers have been identified in birds (Obbard *et al.*, 2009) and are theoretically saturable, although the dose is not known.

8.1.4 Wild Mammals

Refer to Chapter 7 for possible hazards.

8.1.5 Fish

Off-target Gene Silencing

RNAi has only been induced following injection into fish cell cultures or embryos and even so, variable effects were observed. Below are some studies involving RNAi induction in fish with long dsRNAs and siRNAs. It should be noted that all the studies listed below involve injection as a route of exposure, which would not be expected to occur with the use of exogenously applied products.

Long dsRNAs

- Wargelius *et al.* (1999) injected dsRNA targeting the genes no tail (ntl), floating head (flh) and pax2.1 into zebrafish embryos at the one to two-cell stage and found that only a fraction of the embryos had developed the hypothesized gene-specific defects. They noted that in comparison to results from *Drosophila* their treatments in the zebrafish produced a much larger proportion of embryos with non-specific defects.
- Li Y *et al.* (2000) observed 35% full silencing and 53% partial silencing after injecting dsRNAs against ntl into zebrafish embryos (Schyth *et al.,* 2008).
- Oates *et al.* (2000) injected dsRNA corresponding to the T-box gene tbx16/spadetail (spt) into early wild-type zebrafish embryos and observed non-target silencing.
- Zhao *et al.* (2001) injected 7.5–30 pg/embryo of dsRNAs targeting the maternal gene *poull-1*, the transgene GFP, and an intron gene *terra* all into zebrafish embryo and observed various nonspecific defects.
- Mangos *et al.* (2001) injected dsRNA that silenced the *RanBP1*[27] gene into zebrafish embryos, and observed augmented mortality rate and a high frequency of defects.
- Hsieh & Liao (2002) injected dsRNAs silencing endogenous mAChR synthesis into zebrafish embryos and observed almost 100% knockdown at 56 hour post-fertilization.
- Acosta *et al.* (2005) injected dsRNA targeting Myostatin[28] into zebrafish and observed increased body mass with increasing dose.

It has previously been proposed that the conflicting results from the first RNAi studies in fish may have resulted from differences in the dsRNA doses per embryo and differences in the microinjection procedure used and/or activation of interferon response (Schyth, 2008).

siRNAs

- Boonanuntanasarn *et al.* (2003) injected siRNAs targeting GFP into rainbow trout *Onchorynchus mykiss* embryos and were able to reduce the number of strongly fluorescent by 60%. Injection of siRNA sequences with non-perfect match to the GFP mRNA (four mismatches) was not able to reduce the number of fluorescent embryos.
- Schyth *et al.* (2007) delivered naked and polycationic liposome-formulated siRNAs to target the envelope glycoprotein of the fish pathogenic rhabdovirus viral hemorrhagic septicemia virus (VHSV) via intraperitoneal injection. Reduced mortality of virus-challenged fish was observed. Although the delivery method seemed to work, the formulated siRNAs also elicited an interferon response.

Machinery factors that may contribute to off-target silencing:

[27] Ran binding protein 1, a regulator of the Ran gene involved in nucleo-cytoplasmic transport.

[28] Myostatin is a member of the transforming growth factor-β (TGF-β) family that functions as a negative regulator of skeletal muscle development and growth.

Environmental and Systemic RNAi

SID-1 protein homolog ScSidT2 gene is present in fish (Ren *et al.*, 2011) and will likely act as a channel allowing intercellular movement of dsRNA.

Amplification Mechanisms

RdRP pathways are not present (Obbard *et al.*, 2009); however, it is not known if other amplification pathways are present.

Immunostimulation

It is not clear how the immune systems of non-mammal organisms will react to an influx of small RNAs nor is it known how this immunostimulation will affect the fitness of non-target organisms (EPA SAP, 2014). Type-1 interferon stimulation had been observed when long dsRNAs were injected into fish (Masycheva *et al.*, 1995). Interferon activity has also been shown in the zebrafish embryo at 24 h post-fertilization (Schyth *et al.*, 2008). Injection of formulated siRNAs in Schyth *et al.* (2007)'s study elicited an interferon response.

Saturation of Machinery

When Gruber *et al.* (2005) injected 50 µM siRNAs into zebrafish embryos, abnormal morphogenesis relating to incomplete production of miRNAs was observed. This led the researchers to hypothesize that the observation was related to the competition of exogenous and endogenous dsRNA for components of the RNAi machinery.

Dicers are present (Figure 10) (Obbard *et al.*, 2009) and are theoretically saturable, although dose is unknown.

8.1.5 Non-Arthropod Invertebrates

Off-target Gene Silencing

RNAi silencing effects, triggered by either dsRNA or siRNAs, have been observed in plant parasitic nematodes. There is published data for the successful *in vitro* RNAi silencing of more than 40 plant parasitic nematode genes representing nine species within five genera (Lilley *et al.,* 2012).

Machinery factors that may contribute to off-target silencing:

Environmental and Systemic RNAi

Caenorhabditis elegans has evolved mechanisms for diet and dermal uptake of exogenous dsRNA (Whangbo and Hunter, 2008); environmental RNAi has also been well described in flatworms. In the planarian *Dugesia japonica*, gene silencing occurred after the soaking of animals in a dsRNA solution (Orii *et al.,* 2003). The soaking method was effective for genes expressed in cells not in direct contact with the environment (e.g., in the eye, which is located in the mesenchyme inside the body) (Whangbo and Hunter, 2008). The silencing effect of the dsRNA has also occurred in newly regenerated tissues, indicating that planaria are capable of performing both systemic and environmental RNAi (Whangbo and Hunter, 2008). After soaking *Bursaphelenchus xylophilus* in a >2 mg/ml dsRNA solution targeting a cellulase gene (Bx-eng-1) for 24 hours, gene knockdown was observe and the number of F1 generation offspring was reduced significantly (Cheng *et al.*, 2010).

dsRNA delivery via bacterial feeding can trigger RNAi silencing in the planarian *Schmidtea mediterranea* (Newmark *et al.,* 2003). Gene silencing was observed as early as 1–2 days after the third feeding, and

the effects were observed up to 24 days after feeding (Whangbo and Hunter, 2008). It was said that the inhibition was specific and could target genes in a variety of tissue types.

Amplification Mechanisms

In nematodes, RNAi amplification is initiated by RdRPs (Figure 11). The RDE-10/RDE-11 complex is essential for the amplification of RNAi in *C. elegans* by promoting secondary siRNA accumulation (Zhang C *et al.*, 2012). Transitive silencing may occur (see the above Arthropods section for more information).

Immunostimulation

It is not clear how the immune systems of non-mammal organisms will react to an influx of small RNAs nor is it known how this immunostimulation will affect the fitness of non-target organisms (EPA SAP, 2014)

Saturation of Machinery

Dicers are present (Obbard *et al.*, 2009) and are theoretically saturable, although the dose is not known. Dalzell *et al.* (2009) hypothesized that the observation in their experiment was due to the saturation of rate-limiting components of the RNAi pathway. In the experiment, inhibitory effects in *Meloidogyne incognita* (*M. incognita*) increased when the dsRNA dose increased from 0.1 mg/ml to 1 mg/mL. However, in *Globodera pallida*, the 10-fold dsRNA dose increase did not significantly affect the inhibitory effects.

Bakhetia *et al.* (2008) observed inhibitory reduction when *Heterodera glycines* was exposed in combination (rather than individually) to two distinct dsRNAs aimed at silencing two different genes expressed in the dorsal pharyngeal gland cell. This result was thought to be due to the competition between siRNAs for RISC binding.

8.1.6 Microorganisms

Off-target Gene Silencing

RNAi is predominately a eukaryotic pathway. As mentioned in Chapter 7.2, bacteria do not have homologous RNAi machinery but they do have their own mechanism to recognize invading DNAs and RNAs. Initial studies in *Paramecium* established that direct dsRNA injection could lead to loss-of-function phenotypes in microorganisms (Ruiz *et al.,* 1998). Subsequently, feeding dsRNA-expressing *E. coli* to *Paramecium* also generated complete loss-of-function phenotypes for several different target genes (Galvani and Sperling, 2002). In *Candida albicans*, hyphae formation was significantly reduced by EFG1[29] siRNA at concentrations of 1 µM, 500 nM and 100 nM (Moazeni *et al.,* 2012). Gene expression of EFG1 was supressed effectively at 1 µM. In *Aspergillus fumigatus* and *Aspergillus nidulans* , introduction of 10 – 226 nM siRNAs into germinating spores induced sequence-specific gene silencing at least 72 hours after treatment (Jochl *et al.*, 2009; Khatri and Rajam, 2007; Barnes *et al.*, 2008).

In fungi, the mechanism of quelling is generally believed to be equivalent to RNAi in animals because core RNA silencing components such as Dicer, Argonaute, and RdRP genes are used in all of these pathways (Quoc and Nakayashiki, 2015). However, besides these common components, several additional genes in the quelling pathway have also been identified in *Neurospora crassa* (i.e., QDE-3, a DNA helicase). The mechanisms of RNA silencing are conserved in most fungal species with a few exceptions such as *Candida tropicalis, Candida albicans, Candida lusitaniae, Saccharomyces cerevisiae,*

[29] Efg1 is essential for hyphal development in the *Candida albicans*. Efg1 is a transcription factor that can interact specifically with the E box. Source: [HYPERLINK "http://jb.asm.org/content/183/13/4090.full"]

and *Ustilago maydis* (Nakayashiki *et al.*, 2006). Interestingly, comparative phylogenetic analysis shows that numbers of Dicer, Argonaute, and RdRP genes vary significantly among fungal species, suggesting that RNA silencing pathways have diversified in the evolution of fungi. A summary of RNA silencing in fungi and fungus-like organisms is available in Appendix IV.

Machinery factors that may contribute to off-target silencing:

Environmental and Systemic RNAi

Environmental RNAi also exists outside of the animal kingdom. Environmental RNAi by soaking has been demonstrated in the human pathogen *Entamoeba histolytica* (Vayssié *et al.*, 2004). In the study, siRNAs targeting the γ-tubulin gene was added to a 50% confluent *E. histolytica* culture at a final concentration of 10 µg/mL. Highly specific and efficient silencing of the γ-tubulin gene was observed in the form of the disruption of microtubule organization. The *E. histolytica* genome does not appear to contain SID-1 or SID-2 homologs, suggesting that these organisms have evolved an independent mechanism for environmental RNAi.

Amplification Mechanisms

RdRP amplification pathways are not present in bacteria (EPA SAP, 2014); however, other amplification pathways may be present. Fungal RdRP amplification pathways are present (Calo *et al.*, 2012). Transitive silencing may occur (see the above Arthropods section for more information).

Immunostimulation

It is not clear how the immune systems of non-mammal organisms will react to an influx of small RNAs nor is it known how this immunostimulation will affect the fitness of non-target organisms (EPA SAP, 2014).

Saturation of Machinery

No studies found, however, saturation of machinery is theoretically possible.

8.1.7 Plants

Off-target Gene Silencing

In plants, as few as 14 nucleotides of sequence complementarity between siRNA and mRNA can lead to the inhibition of gene expression (Xu *et al.*, 2006; Jackson and Linsley, 2004; Senthil-Kumar *et al.*, 2007; Qiu *et al.*, 2005; Jackson *et al.*, 2006).

Machinery factors that may contribute to off-target silencing:

Environmental and Systemic RNAi

In plants, systemic RNAi is widespread as it is important in restricting viral infection. Studies of plant-specific RNA silencing and trafficking indicate that the mobile RNAi signals involved entail two distinct pathways (Nazim Uddin and Kim, 2013):

1. plasmodesma-mediated cell-to-cell movement. This movement can be subclassified into limited and extensive. Limited movement occurs between 10–15 cells where RDR6 mediated amplification is not required, while extensive movement is more than 10–15 cells, where RDR6 mediated amplification is required.
2. phloem-mediated systemic movement. This movement occurs over the course of days, requiring high amounts of target transcripts for the reception of silencing signal over long distances.

Proteins such as DCL4, NRPD1a[30]/PolIVa[31], RNA-dependent RNA polymerase 2 and CLSY1[32] have been demonstrated to be involved in an independent cell-to-cell transport pathway during RNA silencing (Nazim Uddin and Kim, 2013).

As mentioned previously, San Miguel and Scott (2015) demonstrated that dsRNA in water can be taken up the petioles and was effective in producing RNAi effects. The authors also showed that dsRNA did not move systemically after foliar application.

Amplification Mechanisms

RdRP amplification pathways are present in plants (Figure 11) however, there are uncertainties regarding whether exogenously applied dsRNA will amplify within living plant tissue once it has been absorbed and if so, the degree of amplification (EPA White Paper, 2013). Transitive silencing may occur (see the above Arthropods section for more information).

Immunostimulation

It is not clear how the immune systems of non-mammal organisms will react to an influx of small RNAs nor is it known how this immunostimulation will affect the fitness of non-target organisms (EPA SAP, 2014)

Saturation of Machinery

No studies were found, however, the saturation of machinery is theoretically possible.

For additional information on each taxon, Koch and Kogel (2014) provided a summary of RNAi in several agricultural pests (Appendix V).

[30] A subunit of RNA polymerase IV
[31] a nuclear RNA polymerase IVa
[32] SNF2 domain-containing protein CLASSY: a SNF2 domain-containing protein

Appendix

Appendix I: Approved GM food/feed using RNAi-based technology

International regulatory authorities (including Canada) had approved some RNAi-based GM plants, although the exact mode of action(s) may not been fully elucidated at the time of the approval. Below is a partial list, for a complete list of approved food/feed, please consult the [HYPERLINK "http://www.isaaa.org/gmapprovaldatabase/"].

Crop	Event	Gene introduced	Gene source	Product / Function	Authorized Country(ies)
Apple	OKA-NBØØ1-8; OKA-NBØØ2-9[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=393&Event=GD743"]	PGAS PPO	*Malus domestica* - Apple	dsRNA from the suppression transcript is processed into small interfering RNAs (siRNAs) that direct the cleavage of the target mRNA through sequence complementarity and suppresses PPO resulting in apples with a non-browning phenotype.	Canada, 2015[1,2,3] USA, 2015[1,2,3]
Alfalfa	MON-ØØ179-5	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=99&Gene=ccomt%20(inverted%20repeat)"]	alfalfa	Reduces content of guaiacyl (G) lignin. dsRNA that suppresses endogenous S-adenosyl-L-methionine: trans-caffeoyl CoA 3-O-methyltransferase (CCOMT gene) RNA transcript levels via the RNA interference (RNAi) pathway	Canada, 2014[1,2,3] Australia, 2014[1] New Zealand, , 2014[1] USA, 2013[1,2], 2014[3]
Bean	EMB-PVØ51-1	ac1 (sense and antisense)	Bean Golden Mosaic Virus (BGMV)	sense and antisense RNA of viral replication protein (Rep) produced; no functional viral replication protein is produced. /Inhibits the synthesis of the viral replication protein of the Bean Golden Mosaic Virus (BGMV), thereby conferring resistance to the BGMV	Brazil, 2011[1,2,3]
Corn	MON-87411-9	[HYPERLINK "https://www.isaaa.org/gmapprovaldata	Western Corn Rootworm (*Diabrotica*	RNAi interference resulting to down-regulation of the function of the targeted Snf7 gene leading to Western Corn	USA, 2014[1,2] Japan, 2014[3] (Last updated Feb 11, 2015)

		base/gene/default.asp?GeneID=104&Gene=dvsnf7"]	*virgifera virgifera*)	Rootworm mortality. double-stranded RNA transcript containing a 240 bp fragment of the WCR Snf7 gene	
Potato	SPS-ØØE12-8; SPS-ØØE24-2; SPS-ØØE24-2; SPS-ØØF37-7; SPS-ØØH37-9; SPS-ØØH50-4; SPS-ØØØJ3-4; SPS-ØØJ55-2	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=105&Gene=asn1"]	*Solanum tuberosum*	Generates with (9) double stranded RNA that triggers the degradation of Asn1 transcripts to impair asparagine formation	USA, 2014[1,2,3]
		[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=108&Gene=pPhL"]	*Solanum tuberosum*	Generates with (16) double stranded RNA that triggers the degradation of PhL transcripts to limit the formation of reducing sugars through starch degradation	
		[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=106&Gene=ppo5"]	*Solanum tuberosum*	Generates with (8) double stranded RNA that triggers the degradation of Ppo5 transcripts to block black spot bruise development	
		[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=107&Gene=pR1"]	*Solanum tuberosum*	Generates with (15) double stranded RNA that triggers the degradation of R1 transcripts to limit the formation of reducing sugars through starch degradation	
Soybean	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=177&Event=MON87705	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=59&Gene=fatb1-A%20(sense%20and%20antisense%20seg	Soybean	Production of FATB enzymes or acyl-acyl carrier protein thioesterases is suppressed by RNAi.	Canada 2011[1,2,3] [HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=177&Event=MON87705"]

	"]	ments)"]			
		[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=60&Gene=fad2-1A%20(sense%20and%20antisense)"]		Production of delta-12 desaturase enzyme is suppressed by RNAi	
	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=286&Event=MON87705%20x%20MON89788"]	fatb1-A	Soybean	See above	Mexico 2012[1] South Korea 2013[1], 2014[2] Taiwan 2014[1] (expires 2019)
		[HYPERLINK "http://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=60&Gene=fad2-1A%20(sense%20and%20antisense)"]		See above	
	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=168&Event=DP305423"]	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=97&Gene=gm-fad2-1%20(partial%20sequence)"]	soybean	Expression of the endogenous fad2-1 gene encoding omega-6 desaturase enzyme was suppressed by the partial gm-fad2-1 gene fragment.	Canada 2009[1,2,3] [HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=168&Event=DP305423"]
	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=171&Event=2	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=57&Gene=gm-fad2-1%20(silencing%20locus)"]	soybean	Production of endogenous delta-12 desaturase enzyme was suppressed by an additional copy of the gm-fad2-1 gene via a gene silencing mechanism.	Canada 2000[1,2,3] [HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=171&Event=260-05%20(G94-

	60-05%20(G94-1,%20G94-19,%20G168)"]				1,%20G94-19,%20G168)"], as Australia withdrawn its approval for food in 2011 for commercial reasons
Tobacco	Vector 21-41	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=75&Gene=NtQPT1%20(antisense)"]	*Nicotiana tabacum*	antisense RNA of quinolinic acid phosphoribosyltransferase (QPTase) gene; no functional QPTase enzyme is produced.	USA, 2002[3]
Tomato	SYN B SYN Da	pq (sense or antisense)	*Lycopersicon esculentum*	No functional polygalacturonase enzyme is produced (transcription of the endogenous enzyme is suppressed by a gene silencing mechanism. Inhibits the production of polygalacturonase enzyme responsible for the breakdown of pectin molecules in the cell wall, and thus causes delayed softening of the fruit.	USA 1994[1,2,3] Mexico 1996[1]
	SYN F				Canada 1996[1] +2
	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=178&Event=FLAVR%20SAVR%E2%84%A2"]	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=61&Gene=pg%20(sense%20or%20antisense)"]	*Lycopersicon esculentum*		Canada 1995[1] USA 1994[1,2,3] Mexico 1995[1]
	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/event/default.asp?EventID=186&Event=Huafan%20No%201"]	[HYPERLINK "https://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=65&Gene=anti-efe"]	*Lycopersicon esculentum*	No functional ACO enzyme is produced; Antisense RNA of 1-amino-cyclopropane -1-carboxylate oxidase (ACO) gene. Causes delayed ripening by suppressing the production of ethylene via silencing of the ACO gene that encodes an ethylene-forming enzyme	China 1997[1,2,3]

[1]Food, [2]Feed, [3]Cultivation

Appendix II: Comparison of the siRNA and miRNA pathway components in mammals, plants and insects based on the know models in humans, *Arabidopsis thaliana* and *Drosophila*.

Table 1. Comparison of the siRNA pathway components in mammals, plants and insects based on the know models in humans, *Arabidopsis thaliana* and *Drosophila*.

Component	Humans	Plants	Insects
RNase	Dicer	Dicer-like (DCL) endonucleases 2 & 4	Dicer 2 (Dcr-2)
RNase associated proteins	TAR RNA-binding protein (TRBP) and a protein activator of protein kinase PKR (PACT)	Double-stranded-RNA-binding protein 4 (DRB4)	Loquaciousin (loqs) and R2D2
siRNA Methylase	Not methylated	HUA enhancer 1 (HEN1)	Not methylated
Argonaute at the center of RISC	AGO2	AGO1	AGO2

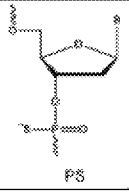
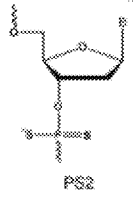
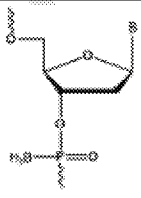
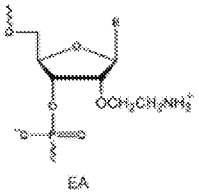
Table 2. Comparison of the miRNA pathway components in mammals, plants and insects based on the known models in humans, *Arabidopsis thaliana* and *Drosophila*.

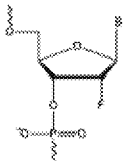
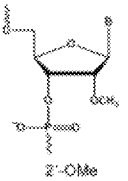
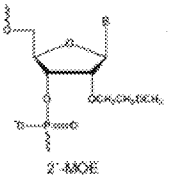
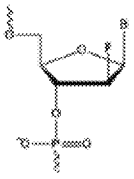
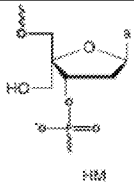
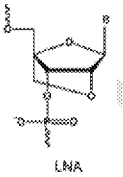
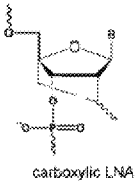
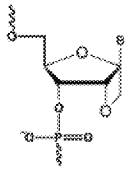
Component	Humans	Plants	Insects
Polymerase	Pol II	Pol III	Pol II
pre-miRNA RNase	Drosha	none	Drosha and Pasha
Exported by	Exportin5	Hasty	Exportin5
pre-miRNA RNase	Dicer	DCL-1	Dicer 2 (Dcr-2)
pre-miRNA RNase associated proteins	TAR RNA-binding protein (TRBP) and a	double-stranded RNA-binding protein 1 (HYL1)	Loquaciousin (loqs)

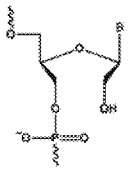
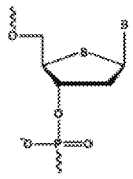
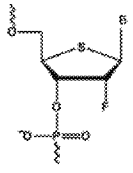
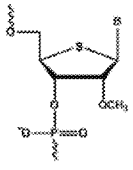
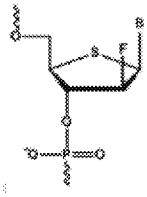
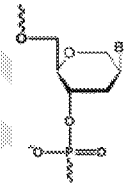
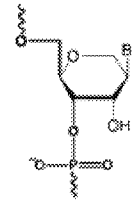
	protein activator of protein kinase PKR (PACT)		
siRNA Methylase	Not methylated	HUA enhancer 1 (HEN1)	Not methylated
Argonaute at the center of RISC	AGO2	AGO1	AGO2
siRNA Methylase	Not methylated	HUA enhancer 1 (HEN1)	Not methylated

Appendix III: Common chemical modification of siRNAs

(Bramsen and Kjems, 2011; Bramsen and Kjems, 2012; Rettig and Behlke, 2012)

Modification		Advantages	Disadvantages
Phosphothioate	 PS	Increases stability and uptake	Reduces silencing, can have toxic side-effects
Phosphodithioate	 PS2	Increases potency and nuclease resistance	can have toxic side-effects
Boranophosphate		Enhances nuclease resistance and stability	
2'-aminoethyl	 EA	Enhances nuclease resistance	

2'-fluoro		Enhances nuclease resistance, among the best tolerated modification	
2'-*O*-methyl		Enhances nuclease resistance and stability, reduces immunostimulation and off-target effects (most commonly used)	
2'-*O*-methoxyethyl		Enhances nuclease resistance and stability, useful for duplex asymmetry	Only tolerated at certain positions
2'-deoxy-2'-fluoro-β-d-arabinonucleic acid		Increases stability and potency	
4'-*C*-hydroxymethyl-DNA		Enhances nuclease resistance	
Locked nucleic acid		Increases thermodynamic stability, enhances nuclease resistance	Has observed toxic side-effects in mice, extensive use reduces potency
2', 4'-carbocyclic-LNA-locked nucleic acid		Increases thermodynamic stability	Has observed toxic side-effects in mice
Oxetane-LNA		Increases thermodynamic stability	

Unlocked nucleic acid	 UNA	Enhances biostability, prevents off-targeting, increases potency	Destabilizes and can affect annealing
4'-thioribonucleis acid	 4'-S	Enhances nuclease resistance, target affinity and potency	Only tolerated at certain positions
2'-deoxy-2'-fluoro-4'-thioribonucleic acid	 F-SRNA	Enhances nuclease resistance, target affinity and potency	
2'-*O*-Me-4'-thioribonucleic acid	 Me-SRNA	Enhances nuclease resistance and silencing duration	
2'-fluoro-4'-thioarabinocleic acid	 4'S-FANA	Increases stability and potency	Toxicity is not well studied, only tolerated at low levels
Hexitol nucleic acid	 HNA	Enhances thermostability, nuclease resistance and silencing duration	
Altritol nucleic acid	 ANA	Enhances thermostability, nuclease resistance and potency	

Appendix IV - RNA silencing in fungi and fungus-like organisms

(Quoc and Nakayashiki, 2015)

Fungal species	RNAi trigger	RNAi target	Transformation
Ascomycota			
Neurospora crassa	Homologous transgene IR[a]	albino-1 (*al-1*) and albino-3 (*al-3*)	PEG
Cladosporium fulvum	Homologous transgene	Hydrophobin (*Hcf-1*)	PEG
Magnaporthe oryzae	IR	*GFP, PKS, MPG1,*	PEG
Venturia inaequalis	IR	*GFP, THN*	PEG
Aspergillus fumigatus	IR	*ALB1/PKSP*	PEG
Aspergillus nidulans	IR	*aflR*	PEG
Aspergillus oryzae	Homologous transgene IR	*Cpase O, amyB*	PEG
Aspergillus niger	Homologous transgene	*pdiA*	Lithium acetate
	IR	*xlnR*	PEG
Histoplasma capsulatum	IR	*ADE2*	Electroporation
Fusarium graminearum	IR	*tri6*	PEG
Fusarium solani	IR	*Csn-1*	PEG
Fusarium verticillioides	IR	*Gus*	*Agrobacterium tumefaciens*
Neotyphodium uncinatum	IR	*lolC-2*	Electroporation
Acremonium chrysogenum	IR	*DsRed*	PEG
Penicillium chrysogenum	Convergent transcription	*pcbC*	PEG
Trichoderma harzianum	IR	*erg1*	PEG
Trichoderma asperellum	IR	*TasSwo*	Microprojectile bombardment
Cryphonectria parasitica	IR	*GFP*[b]	PEG
Sclerotinia sclerotiorum	IR	*pph-1, rgb-1*	PEG
Bipolaris oryzae	IR	*PKS*	PEG
Sordaria macrospora	IR	*sdh*	PEG
Collectotrichum gloeosporioides	IR	*PAC1*	PEG
Botrytis cinerea	IR	*bcsod-1*	PEG

Fungal species	RNAi trigger	RNAi target	Transformation
Verticillium longisporum	IR	*Vlaro-2*	*Agrobacterium tumefaciens*
Blastomyces dermatitidis	IR	*CDC11*	*Agrobacterium tumefaciens*
Microsporum canis	IR	*SUB3*	PEG
Basidiomycota			
Schizophyllum commune	IR	*SC15*	PEG
Cryptococcus neoformans	IR	*CAP59*	Electroporation
Coprinus cinereus	IR	*LIM15*	Lithium acetate
Phanerochaete chrysosporium	IR	Mn*SOD1*	Electroporation
Pleurotus ostreatus	IR	*nmp-3*	PEG
Agaricus bisporus	IR	*URA3/CBX*	*Agrobacterium tumefaciens*
Laccaria bicolor	IR	*NR*	*Agrobacterium tumefaciens*
Moniliophthora perniciosa	IR	Mp*PRX1*/Mp*HYD3*	PEG
Zygomycota			
Mucor circinelloides	Homologous transgene	*carB*	PEG
Mortierella alpine	IR	delta12-desaturase	Microparticle bombardment
Oomycota[b]			
Phytophthora infestans	Homologous transgene	*INF1*	PEG[c]
	Homologous transgene	*Pigpa1*	Electroporation
	dsRNA	*gfp*	Lipofectin
Myxomycete (slime mold)[b]			
Dictyostelium discoideum	IR	*beta-gal*	Electroporation
		discoidin gene family	

[a]IR, hairpin RNA or inverted repeat RNA expressing plasmid
[b]Fungus-like organisms
[c]Lipofectin was added to increase transformation efficiency

Appendix V – RNAi in agricultural pests.

Summary of RNAi in agricultural pests (Koch and Kogel, 2014).

Pathogen	Species	Target gene	Target selection	Host plant	Effect/Comments
Insect	*Diabrotica virgifera*	V-ATPase A	cDNA library	Maize	Obvious reductions in root damage
	Helicoverpa armigera	*CYP6AE14* (cytochrome P450)	cDNA library	*Nicotiana benthamiana* and *Arabidopsis thaliana*	Suppressed *CYP6AE14* expression and reduced growth on gossypol-containing diet
				Gossypium hirsutum (cotton)	Enhanced resistance to cotton bollworms
	Myzus persicae	*Rack1* (gut) and*MpC002* (salivary glands)	Homologous genes	*Nicotiana benthamiana* and *Arabidopsis thaliana*	Silenced *M. persicae* produced less progeny
	Nilaparvata lugens	*NlHT1* (hexose transporter), *Nlcar* (carboxypeptidase), *Nltry* (trypsin-like serine protease)	cDNA library	*Oryza sativa* L. (rice)	Reduction of targeted gene transcripts in the midgut; lethal phenotypic effects after dsRNA feeding were not observed
	Helicoverpa armigera	*CYP9A14* (cytochrome P450 monooxygenases)	Known functional gene	*Gossypium hirsutum* (cotton)	Reduced the larval tolerance to the insecticide deltamethrin
		EcR (ecdysone receptor)	Known functional gene	*Nicotiana tabacum* (tobacco)	Resistance to *H. armigera*; EcR dsRNA also confers resistance to another lepidopteran pest, *Spodoptera exigua*
		CYP6AE14 and *GhCP1* (cysteine protease)	Known functional gene	*Gossypium hirsutum* (cotton)	Cotton plants co-expressing dsRNA and cysteine protease exhibit enhanced bollworm resistance
		HaHR3 (moult-regulating transcription factor)	Known functional gene	*Nicotiana tabacum* (tobacco)	Developmental deformity and larval lethality
	Sitobion avenae	*CbE E4* (carboxylesterase)	Homologous genes	*Triticum aestivum* (wheat)	Reduced progeny production and reduced resistance to phoxim insecticides

Pathogen	Species	Target gene	Target selection	Host plant	Effect/Comments
Parasitic nematodes	*Meloidogyne incognita*	Splicing factor and integrase	Orthologous genes, conserved functions	*Nicotiana tabacum*(tobacco)	Resistance
	Meloidogyne javanica	*MjTis11* (transcription factor)	Known functional gene		Down-regulation of*MjTis11* did not result in a lethal phenotype
	Meloidogyne species: *M. incognita*, *M. javanica*, *M. arenaria*, and *M. hapla*	*16D10* (parasitism gene)	cDNA library, homologous genes	*Arabidopsis thaliana*	Resistance effective against the four major RKN species
	Heterodera glycines	*MSP* (major sperm protein)	cDNA library	*Glycine max* (soybean)	Development of SCN females and number of eggs per cyst were reduced
	Heterodera schachtii	*B05*, *4G06*, *8H07* and*10A06* (parasitism genes)	cDNA library	*Arabidopsis thaliana*	Reduction in the number of mature females
	Heterodera glycines	*Cpn-1*, *Y25* and *Prp-17* (reproduction or fitness-related genes)	Homologous genes	*Glycine max* (soybean)	Suppression comparable to conventional resistance
	Meloidogyne incognita	*Mi-Rpn7* (essential for the integrity of 26S proteasome)	Homologous genes	*Glycine max* (soybean)	Reduced motility and infectivity; no complete resistance
		Mi8D05 (parasitism gene)	Previously identified	*Arabidopsis thaliana*	Up to 90% reduction in infection by *M. incognita*
		flp-14 and *flp-18* (FMRF amide-like peptide genes)	Homologous, known functional genes	*Nicotiana tabacum* (tobacco)	50%–80% reduction in infection
	Pratylenchus vulnus	*Pv010* (spliceosome subunit)	Orthologous gene	Walnut	Reduced nematode infection
	Meloidogyne incognita	*16D10* (parasitism gene)	Known functional gene	*Vitis vinifera* (grape)	Less susceptibility
	Heterodera glycines	*HgALD* (aldolase)	Previously identified	*Glycine max* (soybean)	Decrease in the number of mature SCN females

Pathogen	Species	Target gene	Target selection	Host plant	Effect/Comments
Parasitic plants	*Striga asiatica*	Fatty acid—aromatic amino acids—and AMP biosynthesis, vacuole morphogenesis	Herbicides target	Maize	No resistance; some differences in *Striga* growth rate
	Orobanche aegyptiaca (broomrape)	*M6PR* (mannose 6-phosphate reductase)	Previously identified	Tomato	Significant increase in the percentage of dead *O. aegyptiaca* tubercles on the transgenic tomato plants
	Triphysaria versicolor	*GUS* (reporter gene)	Proof of concept	Lettuce, *Triphysaria*, *Arabidopsis*	GUS silencing; proof of concept
	Cuscuta pentagona	*STM* (SHOOT MERISTEMLESS)	Known functional gene	*Nicotiana tabacum* (tobacco)	Silencing disrupts dodder growth
Bacteria	*Agrobacterium tumefaciens*	*iaaM* and *ipt* (oncogenes)	Known functional gene	*Arabidopsis thaliana* and *Lycopersicon esculentum*	Transformed plants retained susceptible to *Agrobacterium* transformation, but were highly refractory to tumorigenesis
			Known functional gene	Walnut	Crown gall control
Fungi / Oomycetes	*Blumeria graminis* f. sp. *tritici*	*MLO*	Known functional gene	*Triticum aestivum* (wheat)	Resistance
	Phytophthora parasitica var. *nicotianae*	*GST* (glutathione S-transferase gene)		*Nicotiana tabacum* (tobacco)	Resistance; GST negative regulator of defence response
	Blumeria graminis	*Avra10* (effector gene)		*Hordeum vulgare* (barley) and *Triticum aestivum* (wheat)	Reduced fungal development in the absence of the matching resistance gene *Mla10*
	Fusarium verticillioides (= *F. moniliforme*)	*GUS* (reporter gene)	Proof of concept	Tobacco (cv Xanthi)	GUS silencing; proof of concept
	Puccinia	*PSTha12J12*	cDNA library	*Hordeum vulgare*	No obvious reductions in

Pathogen	Species	Target gene	Target selection	Host plant	Effect/Comments
	striiformis f. sp. *tritici* or *P. graminis* f. sp. *tritici*	(haustorial Pst transcript)		(barley) and *Triticum aestivum* (wheat)	rust development or sporulation
	Phytophthora parasitica	*PnPMA1* (H^+-ATPase) and *GFP* (reporter gene)	Previously identified	*Arabidopsis thaliana*	Not sufficient; No reduction in GFP and PnPMA1 transcripts
	P. triticina, P. graminis and P. striiformis	*PtMAPK1* (MAP kinase), *PtCYC1* (cyclophilin) and *PtCNB* (calcineurin B)	Functional orthologs	Wheat	Disease suppression, compromising fungal growth and sporulation
	Fusarium graminearum	*CYP51A*, *CYP51B* and *CYP51C*	Fungicides target	*Arabidopsis thaliana* and *Hordeum vulgare* (barley)	Resistance

Summary of studies to identify or validate insecticide target genes by RNAi (Kim Y *et al.,* 2015).

Insecticide target	Insect	Suppression of transcript (%)	Insecticide treatment
AChE 1 & 2	*Plutella xylostella*	7–34	NT[HYPERLINK "http://www.science \l "tn0010"]
AChE 1 & 2	*Helicoverpa armigera*	NA[HYPERLINK "http://www.sciencedirect.com/science/article/pii/S0048357515000036" \l "tn0015"]	NT
AChE 1 &	*Tribolium*	92–95	Carbaryl, carbofuran,

Insecticide target	Insect	Suppression of transcript (%)	Insecticide treatment
2	*castaneum*		
AChE 1 & 2	*Blattella germanica*	95–97	Chlorpyrifos, lambda-
AChE 1 & 2	*Chilo suppressalis*	50–70	NT
nAChR-α6	*Tribolium castaneum*	Approx. 50	Spinosad
nAChR-α6	*Drosophila melanogaster*	25–44	Spinosad
$GABA_A$-R	*Drosophila melanogaster*	50	NT
RyR 1 & 2	*Leptinotarsa decemlineata*	35–55	Chlorantraniliprole
RyR 1 & 2	*Sogatella furcifera*	78–82	Chlorantraniliprole

Insecticide target	Insect	Suppression of transcript (%)	Insecticide treatment
APP	*Ostrinia nubilalis*	38	Cry1Ab
APN	*Spodoptera litura*	95	Cry1C
Cad	*Spodoptera exigua*	Approx. 80	Cry1Ca

*Insects were not treated with insecticides
[a] Information not available.

Summary of studies to reveal roles of the genes in insecticide detoxification and resistance by RNAi (Kim Y *et al.*, 2015).

Target gene	Insect	dsRNA delivery method	Suppression of transcript (%)	Insectici treatme
CYP321E1	*Plutella xylostella*	Injection	13–54	Chlorant
CarEA1 & A2	*Locusta migratoria*	Injection	86–97	Chlorpyr
CYP409A1 & CYP408B1	*Locusta migratoria*	Injection	99	Deltame
CYP6AE14	*Helicoverpa armigera*	Transgenetic plant	NA[HYPERLINK "http://www.sciencedirect.com/science/article/pii/S0048357515000036" \l "tn0020"]	Gossypc
CYP6BG1	*Plutella xylostella*	Feeding	44–69	Permeth
CarE E4	*Sitobion avenae*	Transgenetic plant	30–60	Phoxim
CarE	*Aphis gossypii*	Feeding	33	Ometho
CarE9 & E25	*Locusta migratoria manilensis*	Injection	NA	Malathic

Target gene	Insect	dsRNA delivery method	Suppression of transcript (%)	Insectici treatme
GSTe1 & m2	*Nilaparvata lugens*	Injection	60–90	Chlorpyr
GSTs5 & u1	*Locusta migratoria*	Injection	NA	Carbaryl malathio chlorpyr
GSTs3	*Locusta migratoria*	Injection	NA	Carbaryl
CYP6AA5	*Aedes aegypti*	Injection, feeding	39–78	Cyperme

*Insects were not treated with insecticides

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